NUTRITIONAL AND IMMUNOLOGICAL STUDIES IN INFLAMMATORY BOWEL DISEASE

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Doctor of Medicine

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DECLARATION

I declare that this thesis has been composed by me and that the work contained within it was performed by me, except where clearly stated otherwise. The entire work was performed while I held a post at the Gastrointestinal Unit, Department of Medicine, Western General Hospital, Edinburgh. The thesis has not been submitted for any other professional qualification.

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ABSTRACT

Nutritional problems are common in inflammatory bowel disease. The pathogenesis is multifactorial and the primary role of the disease and associated immunological abnormalities are difficult to assess. In this thesis I have investigated the direct contribution of inflammatory bowel disease in specific nutritional problems such as osteopenia. I have also evaluated clinically applicable objective measures of different aspects of gut inflammation so that the interface between gut inflammation and nutritional abnormalities may be explored.

Bone mineral density and biochemical parameters of bone metabolism were measured in patients with newly diagnosed inflammatory bowel disease prior to medical therapy or surgery. I established that, at diagnosis, low bone mineralisation was a feature of Crohn's disease but not ulcerative colitis. Disease activity, anatomy of involvement, body mass index, smoking habits, sex, physical activity or biochemical parameters did not account for this difference. Follow-up over a period of one year did not show any further bone loss in spite of steroid therapy. Previous studies which had not shown any difference between Crohn's disease and ulcerative colitis had recruited unselected patients with predominantly long-standing disease. When I studied patients with long-standing inactive inflammatory bowel disease, bone mineral density was equally low in both Crohn's disease and ulcerative colitis. Adolescents with long-standing inflammatory bowel disease were identified as a specially vulnerable group for osteopenia. Since bone is only one of the compartments of the body, I next investigated methods to assess body composition in clinical practice. Simple portable, user-friendly bioelectrical impedance analysis machines have recently become available. I evaluated one such machine by comparing it with a standard bioelectrical impedance machine available in Medical Physics department and with dual energy X-ray absorptiometry, a standard method of measuring lean body mass. The results showed that this machine measured lean body mass as accurately as the more expensive, non-portable machines. Furthermore, measurement of percent lean clearly showed heterogeneity in body composition in subjects with similar body mass indices.

The laboratory aspect of my thesis was to establish methods for clinical analysis of various aspects of gut inflammation using the relatively new, non-

invasive technique of whole gut lavage. The patients drank a polyethylene-glycol-electrolyte solution and the first clear fluid passed per rectum was collected for analysis. Cytology of whole gut lavage fluid proved to be a useful technique for investigation of luminal migration of inflammatory cells and I observed previously unrecognised differences between small bowel and colonic Crohn's disease. A simple assay of granulocyte elastase was a good surrogate marker of neutrophils in whole gut lavage fluid and allowed me to study neutrophil migration into the lumen in samples from a large number of patients stratified according to anatomy, treatment and disease activity. The technique also allowed the study of chemokines involved in cell migration. A number of cytokines could be readily measured in whole gut lavage fluid. Raised interleukin-1β concentration was a feature of active inflammation. Interleukin-8 concentration in whole gut lavage fluid correlated with granulocyte elastase concentration in ulcerative colitis, but not in Crohn's disease. This suggested that other luminal neutrophil chemoattractants such as bacterial peptides might be important in Crohn's disease. I also investigated the role of growth factor peptides such as insulinlike growth factor-1 and transforming growth factor-β in gut fibrosis and found detectable insulinlike growth factor-1 in whole gut lavage fluid to be a feature of Crohn's disease and other intestinal inflammatory conditions associated with fibrosis. These clinically applicable techniques should allow future investigation of the role of immunological disturbances in causing some of the nutritional abnormalities in inflammatory bowel disease.

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LIST OF PUBLICATIONS BASED ON THIS THESIS

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Ghosh S, Drummond HE, Ferguson A. Monitoring of growth and development variables in children with inflammatory bowel disease (IBD) - an improvement in practice. Gut 1994;35(suppl 5):S77.

Ghosh S, Drummond HE, Ferguson A. Morbidity and treatment in Scottish children with IBD - a comparison of two cohorts from '68-'83 and '84-'88. Gut 1994;35(suppl 5):S78.

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ABBREVIATIONS

BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
CD	Crohn's disease
CDAI	Crohn's disease activity index
CoV	Coefficient of variation
DEXA	Dual energy X-ray absorptiometry
Dpd	Deoxy-pyridinoline
ELISA	Enzyme linked immunosorbent assay
GE	Granulocyte elastase
Hcl	Hydrochloric acid
IBD	Inflammatory bowel disease
ICD	International classification of diseases
IFN-γ	Interferon-γ
IGF-1	Insulinlike growth factor-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
LBM	Lean body mass
MHz	Megahertz
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PTI	Powell-Tuck index
Pyd	Pyridinoline
SHIPS	Scottish Hospitals' Inpatient Statistics
TGF-β	Transforming growth factor β
TOBEC	Total body electrical conductivity
TPN	Total parenteral nutrition
25-OH-Vit D	25-hydroxy-vitamin D
UC	Ulcerative colitis
WGLF	Whole gut lavage fluid

SECTION I

Introduction

Literature review

Chapter I

INTRODUCTION

Inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are chronic, incurable diseases of unknown aetiology. A number of components of overall disability associated with IBD can be analysed in a systematic way. These include disease activity, anatomical distribution of destructive chronic inflammation, mechanical obstruction, complications such as abscess or fistula, nutritional status, iatrogenic factors (medical and surgical), psychological and social factors, and non-intestinal involvement. Though most of these factors are inter-dependent, analysis of them individually can be useful in understanding their separate contributions to overall morbidity and their influence on therapeutic plans. This thesis is an attempt to investigate some specific aspects of morbidity in IBD, namely, nutritional status, inflammation and tissue destruction, and their possible inter-relationships.

1.1. Nutrition in IBD

Patients with IBD are at increased nutritional risk for deficiency of calories, proteins, vitamins and minerals (Farmer *et al* 1975, Driscoll *et al* 1978, Beeken 1979). Specific subgroups such as children and adolescents, those with multiple bowel resections, and those with complicated disease such as fistulae, are well recognised to be at particular risk. The effect of CD on nutritional status is more marked than that of UC (Barton *et al* 1990, Markowitz *et al* 1993). Though nutritional deficiencies in IBD are a major concern, the database concerning nutritional status of patients with IBD is relatively modest compared with data on the pathogenic or therapeutic aspects of IBD. In addition, interpretation of the available data can be difficult because many of the studies have methodological shortcomings; some are retrospective with the use of historical controls, others are uncontrolled, and still others concern heterogeneous cohorts of patients. Available studies generally focus on patients from specialised centres with complicated disease. In children with IBD, increased morbidity of children with CD in relation to growth and development delay has been described in studies from our centre

(Barton *et al* 1990, Ferguson *et al* 1994b) and other centres (Hildebrand *et al* 1994, Griffiths *et al* 1993, Markowitz *et al* 1993, Motil *et al* 1993).

1.2. Multifactorial aetiology of malnutrition

Nutritional deficiency in IBD is multifactorial. Diminished intake, malabsorption, increased intestinal losses of protein and blood, medical and surgical therapy and sepsis contribute to malnutrition. Less well recognised is the direct effect of the disease process on metabolism and nutrition; this has been extensively researched in malignant diseases (Hansell *et al* 1986, Fredrix *et al* 1991) and in acquired immunodeficiency syndromes (Grunfeld *et al* 1992, Mildvan *et al* 1992), but comparable studies are lacking in the area of IBD. Understanding the role of the different variables on nutritional status, including the direct effect of the disease itself, is one of the major challenges in this area of IBD management and research.

1.3. Teasing out the contribution of disease process from the effects of medical therapy, surgery and malabsorption.

It is difficult to study the direct effect of IBD on nutritional parameters as there are many confounding variables. Prospective studies on patients carefully stratified according to their medical therapy and surgical resections, anatomy of disease and disease activity are needed to tease out the contribution of a single variable. There are few studies on newly diagnosed patients who probably offer the best opportunity to study the effect of the disease process before medical or surgical therapy. Recruiting and prospectively studying patients at diagnosis may take longer than cross-sectional studies, but the smaller number of variables in matched cohorts make interpretation much easier and valid.

1.4. Children with IBD - a specially vulnerable group

Though studies from specialised centres dealing with complicated patients have established that there is delayed rate of growth and development in a considerable proportion of children with IBD, such data are lacking for unselected, population- based cohorts of children with IBD. In Scotland, serious deficiencies in recording growth and development parameters in IBD children attending NHS hospitals have been highlighted (Barton *et al* 1989a). This makes longitudinal

studies based on retrospective analysis of hospital records difficult. Only an improvement in monitoring resulting from greater awareness will permit longitudinal analysis of growth and development in population-based surveys of juvenile-onset IBD patients.

1.5. Importance of a compartmental approach to nutritional assessment

Traditional methods of assessing nutritional status rely heavily on measurement of weight and height with some additional laboratory parameters. Though this may generally be adequate for clinical monitoring of patients, it is impossible to formulate hypotheses regarding the mechanism of undernutrition or possible metabolic changes unless a more detailed assessment of body composition is performed. Loss of weight may be attributable to loss of both fat and lean, or may be the result of a relatively selective loss from one of the two compartments; the underlying metabolic abnormality is likely to be very different in these groups.

1.6. Investigating the effect of disease-objective methods of assessing various aspects of inflammation

In order to conduct objective studies on the effect of IBD on nutrition, various aspects of inflammation need to be objectively measured and correlated with nutritional parameters. Currently, few methods provide a direct accurate measurement of gut inflammation and its various components in a clinical setting and novel approaches to clinical quantitative measurement of various aspects of gut inflammation are needed.

1.7. Aims of this thesis

The general aim of this thesis was to assess the effect of IBD on specific compartments of the body, such as bone, fat and lean and develop clinical methods to investigate specific inflammatory processes so that any interrelationship between body compartment changes and inflammation may be investigated. I proposed to investigate the hypothesis that CD, a systemic disease with a long premorbid phase, has direct effects on nutritional status unlike UC which is characterized by inflammation localized to colonic mucosa only. The effect of CD on nutrtition is likely to be multifactorial and involve diminished intake, the

effect of inflammation on intermediary metabolism and the effects of diminished physical activity, drugs and surgery. In uncomplicated starvation, fat compartment is selectively depleted first in order to spare muscle protein (Cahill 1970). Pro-inflammatory cytokines such as TNF or IL-1 result in widespead disturbances in intermediary metabolism and as observed in AIDS and in some cancer patients, wasting is owing to loss of body cell mass with little loss of fat (Grunfeld and Feingold 1992). Hence in this thesis, the lean/fat body compartment in patients with IBD was studied. Another organ which in influenced by undernutrtion as well as by inflammatory mediators is the bone compartment. I therefore studied the lean/fat body composition as well as the bone compartment in an effort to detect disturbances that may result from gut inflammation. I also attempted to develop methods to study the gut inflammation in a relatively non-invasive manner.

1.7.1. Development of studies to investigate the direct effect of IBD on bone mineral density

A number of studies have documented osteopenia in IBD (Compston *et al* 1987, Motley *et al* 1988, Pigot *et al* 1992, Clements *et al* 1992). Bone mineral density can be measured objectively and accurately and may provide a distinct compartment to study the direct effect of disease on nutritional status. The metabolic influences on bones are well established and can be readily measured. I therefore chose bone as the compartment to study the direct effect of IBD. I aimed to study newly diagnosed patients prospectively before any therapy or surgery to control for the many important confounding variables mentioned above. Patients with long-standing IBD were also studied to determine the effects of medical and surgical therapy and disease duration.

1.7.2. Development of bedside methods of studying body composition in IBD patients

An ideal method of studying body composition in a clinical setting should be safe, inexpensive, quick, free of hazards such as radiation, accurate and reproducible. None of the currently available methods fulfils all or even most of these criteria and I aimed to evaluate new technology that had the potential to meet these clinical requirements.

1.7.3. Investigation of bone density and growth parameters in adolescents

Since children and adolescents with IBD are specially vulnerable to nutritional problems, I aimed to study them as a separate group regarding growth parameters and bone mineral density. I had to establish first whether monitoring of growth and development parameters in Scottish hospitals had improved sufficiently to permit longitudinal retrospective population-based analysis.

1.7.4. Development and evaluation of laboratory methods of studying specific aspects of gut inflammation

Inflammation is characterised by effector cells and their regulatory mediators. My predecessors in the gastrointestinal laboratories have established the technique of whole gut lavage using a polyethylene glycol-electrolyte solution as an extremely useful method of assessing gut-derived protein loss and bleeding (O'Mahony *et al* 1990, Brydon *et al* 1992, Brydon *et al* 1993). It can be applied to the investigation of intestinal immunity and inflammation and I evaluated the clinical usefulness of whole gut lavage in measuring neutrophil migration into the gut lumen and in studies of the various regulators of inflammation such as cytokines and growth factor peptides in the gut.

1.8. Approach

1.8.1. Study of bone mineral density in newly diagnosed patients with IBD

Dual Energy X-ray Absorptiometry (DEXA) is widely regarded as the most accurate and reproducible method of measuring bone mineral density (Sartoris *et al* 1989, Clements *et al* 1994, Compston *et al* 1995a and 1995b). I utilised the facilities available at the Medical Physics department of the University of Edinburgh to prospectively study a cohort of newly diagnosed patients with IBD and compare CD with UC.

1.8.2. Study of bone mineral density in patients with long-standing IBD

I also recruited specifically a cohort of patients with long-standing inactive disease to compare the effects of confounding variables such as steroid therapy or surgery with the effect of disease in the newly diagnosed cohort.

1.8.3. Pathogenesis of low bone density in newly diagnosed patients

A number of relatively non-invasive methods are now available to assess bone turnover and the activity of osteoblasts. These include the determination of plasma osteocalcin as a marker of osteoblastic activity and urinary pyridinium crosslinks as markers of osteoclast activity. In collaboration with Dr Simon Robins at the Rowett Research Institute in Aberdeen, I have measured these parameters in a separate newly diagnosed cohort of IBD patients to assess the effect of disease on bone turnover. This study is ongoing.

1.8.4. Study on a cohort of normal volunteers and diseased patients to evaluate bedside measurement of body composition.

I evaluated bedside measurement of body composition using a user-friendly machine to measure bioelectrical impedance and compared the results with those obtained by DEXA absorptiometry and with a standard but less user-friendly bioelectrical impedance machine.

1.8.5. Body composition and bone mineral density

DEXA was used to measure bone mineral density and lean and fat mass simultaneously in a well-defined cohort of inflammatory bowel disease patients so that bone density could be assessed in the context of total body composition.

1.8.6. Studies in adolescents with IBD

Children and adolescents with IBD are a specially vulnerable group from the standpoint of nutritional problems. I have reported on bone mineral density in a selected group of complicated IBD and on the growth and development parameters in a community-based study on Scottish patients with juvenile-onset IBD, utilising the Scottish Hospital Inpatients Statistics database (SHIPS) as a powerful epidemiological tool to identify cases.

1.8.7. Study of neutrophil migration into the gut lumen

IBD is characterised by accumulation of neutrophils in the mucosa of the gut followed by migration across the epithelium into the lumen of the gut. I used cytology and granulocyte elastase assay of whole gut lavage fluid specimens to perform a clinical analysis of the usefulness of investigating neutrophil migration

into the gut lumen. The subjects studied included a large, heterogeneous cohort of well characterised IBD patients, other diseased controls and normal controls.

1.8.8. Investigation of gut cytokines and growth factor peptides

An advantage of using whole gut lavage fluid to study cellular migration into the lumen is that chemical mediators involved in chemotaxis can be studied simultaneously. I have analysed the usefulness of investigating chemoattractants such as IL-8 and its stimulators such as IL-1β. The role of growth factor peptides such as IGF-1 and TGF-β in gut fibrogenesis was also studied using whole gut lavage fluid.

1.9. Structure of the thesis

1.9.1. Section I .

This section includes the introduction and a literature review. The review focuses in a large measure on the interface between nutrition and immunity/ inflammation but also gives a selected review of body composition and bone mineral density in IBD. Aspects of inflammation and its mediators in IBD are also reviewed.

1.9.2.Section II.

This section describes the methods I used to study bone mineral density and body composition and biochemical methods of studying calcium homeostasis. It presents the results of my studies on bone mineral density at diagnosis and in patients with long-standing IBD, mechanism of bone loss, evaluation of bedside methods to measure body composition in health and disease and body composition in IBD. The final chapter deals with studies on adolescents with IBD; the results of my studies on bone mineral density and growth are presented. A discussion at the end of this section sums up the implications of my findings.

1.9.3. Section III .

This section describes laboratory work. It describes the development of methods to study neutrophil migration into the gut lumen by cytology and by granulocyte elastase assay of whole gut lavage fluid. Clinically applicable methods of studying cytokine production in the gut mucosa are described, and the development of novel methods of studying gut fibrosis is explained. Results of my studies on stratification of disease by neutrophil migration into the gut lumen and studies on cytokine profile in whole gut lavage fluid are presented. The results of a study on the potential usefulness of assaying growth factor peptides in whole gut lavage fluid to investigate fibrogenesis in the bowel wall are recorded and evaluated. In the final chapter, I have discussed the implications of the laboratory results. An epilogue summarises the new hypotheses I have developed as a result of this work and outlines further studies arising from it that have already been launched.

♦ ♦ ♦

Chapter II

BACKGROUND AND LITERATURE REVIEW

2.1. Analysis of morbidity in patients with inflammatory bowel disease (IBD)

The components of overall disability of patients with IBD may be analysed in a systematic way, and this type of analysis can profoundly influence treatment plans. Several components may be identified, such as extent and distribution of destructive chronic inflammation, disease inflammatory activity, mechanical obstruction, complications such as abscess or fistula, nutritional status, iatrogenic factors (medical or surgical), social and psychological factors and intercurrent illnesses (such as ankylosing spondylitis, gallstones etc.). Methods to measure these various components objectively using clinically applicable tests can greatly assist analysis of IBD and my predecessors have been involved in developing such methods to measure various aspects of gut inflammation and immunity (Ferguson *et al* 1994a). In this thesis, I have measured specific aspects of nutritional status such as bone mineral density and body composition. Furthermore, I have investigated the direct effect of disease on nutrition by studying newly diagnosed patients. In order to study the effect of inflammation on nutrition, I developed and analysed new methods to study various aspects of intestinal inflammation. These provided further information about the effects of anatomy of disease, medical therapy and diagnosis on intestinal immunity and inflammation in IBD. This chapter reviews these various concepts.

2.2. Nutrition in adults and children with IBD

In adult patients with Crohn's disease (CD) weight and body mass index (BMI) are significantly lower than in the general population. This is irrespective of disease localisation or inflammatory activity. Possible incriminating factors include low intake of calories and specific nutrient malabsorption, gut losses of protein and blood, increased

basal- and disease- associated metabolic rates, growth hormone deficiency and impaired metabolic responses to growth hormone (Kirschner 1990). However resting metabolic rate and total daily energy expenditure were normal in a series of stable IBD out-patients (Kushner *et al* 1991). In growth retarded IBD children, growth hormone responses are generally normal (Farthing *et al* 1981) but these patients have low levels of plasma insulinlike growth factor-1 (IGF-1) (Kirschner *et al* 1986) and a similar abnormality of IGF-1 has been reported in extremely malnourished adult IBD patients (Clemmons *et al* 1985). Most reports of growth and development in young patients with IBD have emphasised retardation of sexual maturation and of linear growth (height). Growth delay has been recognised even before the onset of symptoms (Kanof *et al* 1988) and growth spurts after surgery may be transient and unpredictable (Homer *et al* 1977, Castile *et al* 1980, McLain *et al* 1990, Lipson 1990). Since intensive nutritional repletion can reverse linear growth failure in growth retarded children with CD, malnutrition is generally accepted as an important factor. (Seidman *et al* 1987). Growth delay is less of a problem in ulcerative colitis (UC) than in CD. Adults who had developed UC in childhood were found to be of normal weight and BMI (Ferguson *et al* 1994b).

Hence it is well established that malnutrition is a major problem that frequently complicates the course of IBD patients, especially those with CD. The impact of this is very considerable in prepubertal patients (Seidman *et al* 1989). The causes are likely to be many, but several studies have emphasized the fact that inadequate calorie intake is important. Though oral nutritional supplementation with polymeric liquid formulae has yielded disappointing growth results over the long term, intermittent courses of elemental diet therapy may reverse growth failure (Belli *et al* 1988). Elemental diet treatment has several potential effects on the patients with CD, immunological as well as nutritional.

Considerable progress has been made in understanding the wasting syndromes; much information has come from studying patients with malignancies, acquired immune deficiency syndrome (AIDS) or other chronic infections. such as Leishmania or tuberculosis. The roles of cytokines as mediators of the metabolic disorders which lead to wasting are being increasingly recognised. Considerable data

exists on cytokines in IBD (Sartor 1994), but the link between these protein mediators of inflammation produced by lymphocytes, macrophages and many other cells, and the cachexia seen in some patients with CD, is unclear.

One of the central themes to emerge in the last few years has been that there are striking differences from a metabolic standpoint between the wasting in malignant and infectious diseases (exemplified by AIDS) and the wasting seen in uncomplicated starvation. In starvation, fat tissue is selectively depleted in order to spare muscle protein; the muscles waste only after fat stores are exhausted (Kotler *et al* 1985, Cahill 1970, Brennan 1977). In AIDS and in some cancer patients, metabolic disturbances prevent nitrogen sparing but conserve adipose tissue (Kotler *et al* 1989, Brennan 1977). Many of the infections characterised by weight loss are associated with hypertriglyceridemia whereas, in starvation, plasma lipid levels are low. It has long been recognized that the degree of wasting correlates poorly with the infective burden (Beisel 1975) and metabolic changes associated with infection can be reproduced by administering non-viable extracts of bacteria (Kaufman *et al* 1976, Lang *et al* 1985). It thus appears that a component of the host response causes the changes in metabolism, now generally accepted to be proinflammatory cytokines (Grunfeld *et al* 1991).

It must also be noted that there are many ways in which nutrition can affect gut immune state, either as part of a general effect of nutritional state on the host, or in relation to the provision of certain nutrients in the microenvironment of the epithelial and subepithelial tissues (Ferguson 1994c).

2.3. The 'Cachectin' hypothesis

Cerami's group recognized that many of the infections characterised by weight loss are associated with hypertriglyceridemia. The latter may result from decreases in lipoprotein lipase, the enzyme responsible for triglyceride clearance (Beutler 1987). They sought a factor in plasma that might induce both hypertriglyceridemia and wasting by the promotion of adipose tissue catabolism. Supernatants from cultures of activated macrophages were tested and found both to induce weight loss when given daily to rodents *in vivo* and to decrease lipoprotein lipase activity in cultured fat cells *in*

vitro. The 'cachectin' factor responsible for these catabolic effects was purified by a method that used its ability to inhibit lipoprotein lipase and was found to be the cytokine 'tumour necrosis factor' (TNF). Other experiments confirmed that purified recombinant TNF lowers lipoprotein lipase activity, decreases the synthesis of fatty acids and promotes lipolysis in cultured fat cells (Patton *et al* 1986, Grunfeld *et al* 1991,). However interleukin-1 and interferons α, β and γ have these same catabolic effects on cultured fat cells (Keay *et al* 1980, Beutler *et al* 1985). Thus if the ability to promote catabolism in cultured fat cells defines the cachectin factor, then a number of cytokines shares this property. Beutler *et al* in 1985 purified cachectin to homogeneity in cell culture and found it to be a polypeptide hormone with a subunit size of 17KD. Cachectin accounted for 1-2% of the total secretory product of endotoxin activated RAW 264.7 cells (Beutler *et al* 1985), a murine macrophage line, i.e., it occurs in considerable abundance.

The catabolic effects of these cytokines on fat-cell metabolism are not reversed by insulin, a hormone that enhances fat storage, (Grunfeld *et al* 1991, Patton *et al* 1986) suggesting that effects on fat cells *in vitro* may not reflect the disturbances *in vivo,* since cachexia is characterized by decreases in muscle mass whereas the changes in fatty tissue vary (Kotler *et al* 1985,1989). In the cachexia of lymphoma for example, hyperalimentation (which results in hyperinsulinaemia) promotes the storage of fat but does not increase muscle mass (Popp *et al* 1981). Likewise, in patients with AIDS who have severe untreated secondary systemic infections, hyperalimentation produces more effective fat storage than preservation of body cell mass (Kotler *et al* 1990).

2.4. The role of cytokines in wasting in IBD

2.4.1. Cytokines with important metabolic roles

2.4.1.1. Interleukin-1 (IL-1)

IL-1 is produced primarily but not exclusively by macrophages/monocytes and consists of at least two 17KD polypeptides (IL-1 α and β) which are involved in a wide spectrum of immunoinflammatory activities. These include fever, wasting, increased vascular permeability, leukocytosis, lowering of plasma levels of iron and zinc,

induction of acute phase protein synthesis in the liver, release of proinflammatory mediators such as histamine, plasminogen, platelet-activating factor (PAF), eicosanoids, collagen and collagenase, and induction of free oxygen radical production. IL-1 also plays a crucial role in antigen-dependent T-cell activation by providing an essential co-stimulatory signal for these cells to produce lymphokines, particularly B-cell growth factors such as IL-2, IL-4, IL-5, IL-6 and IFN-γ (Bendtzen 1989).

IL-1 has catabolic effects similar to TNF on cultured fat cells (Patton *et al* 1986 Grunfeld 1991 *et al*) and recombinant IL-1 suppresses lipoprotein lipase activity in 3T3-L1 cells (Beutler *et al* 1985). It has been shown to produce anorexia in rats (Hellerstein 1989).

2.4.1.2. Interleukin-6 (IL-6)

IL-1α, IL-1β and TNF are all potent inducers of IL-6 in both macrophage/ monocyte and T cells (Bendtzen 1991a, 1991c). IL-6 may act as an important second messenger of IL-1 and TNF. IL-6 plays a key role in the acute-phase response by inducing increased acute-phase protein synthesis in hepatocytes (Bendtzen 1989). Furthermore, IL-6 stimulates T and B cells, most likely because it increases the responsiveness of these cells to IL-2 (Snick 1990).

2.4.1.3. Tumour Necrosis Factor (TNF)

TNF-α and β are 17KD polypeptides whose genes are located in the major histocompatibility complex (MHC) region (Dunham *et al* 1987, Ragoussis *et al* 1988). TNF-α and β are potent coactivators of T and B cells (Bendtzen 1991c). Elevated circulating and local TNF-α concentrations have been found in many infectious and non-infectious conditions, characterized by macrophage/monocyte and/or T-cell-mediated injury, such as renal allograft rejection.

2.4.1.4. Interferon-γ (IFN-γ)

IFN-γ is a lymphokine that acts as a potent activator macrophage /monocytes. IFN-γ has a molecular mass of 20-25KD. It is produced by T-cells and activates several

important inflammatory cells (Bendtzen 1991a). As mentioned before, it is likely that when circulating monocytes are primed by IFN-γ, synthesis of cachectin (TNF) is greatly augmented.

2.4.2. Cytokines in IBD

Recent reviews (Brynskov *et al* 1992b, Sartor 1994) have summarised the data available on the role of the various cytokines in IBD. These papers have concentrated on the role of the cytokines as inflammatory mediators but far less data exists on the metabolic abnormalities produced by them. Discussed below is the information on the four putative `suspects' of the wasting syndrome regarding their production in IBD.

(1)TNF: Several studies have failed to show significant differences in serum or mucosal TNF-α levels between IBD patients and controls (Hodgson *et al* 1991, Hyams *et al* 1991). However Murch *et al* (1991) suggested that production of TNF-α may be associated with growth failure in children in relapse of colonic IBD. They measured TNF-α concentrations by ELISA in the serum of 31 normal children and during 65 episodes of clinical remission and 54 episodes of relapse in 92 children with chronic IBD. An appreciable rise in TNF-α was found only in children with relapse of UC and of colonic CD. The group of children with small bowel CD in relapse did not show increases of TNF-α. Height velocity was depressed in children with relapse of large bowel CD and UC compared with the equivalent condition in remission and compared with small bowel CD alone.

(2) IL-1: IBD sera lack significant IL-1 activity as measured by either bioassay (Satsangi *et al* 1987), or ELISA (Brynskov *et al* 1991). In patients with active IBD, the production of IL-1 by isolated blood mononuclear cells *in vitro* has been found to be either normal (Miura *et al* 1985) or increased using (Satsangi *et al* 1987, Suzuki *et al* 1991, Hodgson *et al* 1991). At the mucosal level both an increased spontaneous and a lipopolysaccharide-induced production of IL-1β have been consistently demonstrated *in vitro* with mucosal mononuclear cells isolated from surgically removed bowel from patients with active IBD (Mahida *et al* 1989). It has been shown that macrophages are the major source of IL-1 in IBD mucosa (Mahida *et al* 1989). Further studies using endoscopic mucosal biopsy specimens confirmed these findings (Ligumski *et al* 1990,

Brynskov *et al* 1992a) and showed that elevated mucosal levels of IL-1β correlate with the degree of histologic activity (Brynskov *et al* 1992a). These data indicate that increased IL-1 production and release occurs both *in vitro* and *in vivo* at the mucosal level in patients with active IBD. In spite of some similarities in the metabolic effects of IL-1 and TNF, no studies have looked into the role of IL-1 in growth failure or wasting in IBD. Hellerstein *et al* (1989) showed that IL-1 induces anorexia in rats. The anorexic effect appears to be mediated by prostaglandins since pre-treatment with ibuprofen completely blocked it. More interestingly, a fish oil based diet abolished the anorexic effect of IL-1, while corn oil or chow diets had no effects. Placed in a wider context, these findings add support to the concept that nutrients and inflammation are intimately intertwined in a complex bi-directional fashion mediated by cytokines. Thus dietary protein and source of fatty acids may alter IL-1 release (Hoffman-Goetz *et al* 1979), dietary fats may alter cytokine actions, carbohydrates, in theory, may be involved as signals in their anorexic effects (Baile *et al* 1970, Van Itallie *et al* 1977, Russek 1981, Sullivan *et al* 1985, Langhans *et al* 1985), and inflammatory mediators not only alter metabolism of all classes of substrates (Feingold *et al* 1987, Tredget *et al* 1988) but reduce their overall intake by causing anorexia. From a clinical perspective, perhaps most interesting is the ability of a dietary supplement (fish oil) to diminish anorexia caused by IL-1. This could be a potentially simple and safe intervention in patients experiencing anorexia and weight loss in association with an inflammatory illness. When 3g/d of EPA in fish oil capsules was added to normal Western diets of human volunteers, a 50% reduction in IL-1 production occurred when their blood leukocytes were stimulated *ex vivo* (Endres *et al* 1989).

(3) IL-6: Mahida *et al* (1991) detected high circulating concentrations of IL-6 in active CD but not UC. Gross *et al* (1992) confirmed increased serum IL-6 concentrations in patients with CD compared with patients with UC and controls. There was no correlation between serum IL-6 concentrations and clinical activity indices, but this could be explained by the short circulatory lifetime and rapid hepatic clearance of IL-6 from portal venous blood. Acute phase proteins induced by IL-6 have a longer circulatory lifetime and several studies have shown that they are significantly correlated with clinical activity indices (Mahida *et al* 1991, Gross *et al* 1992). Both

mononuclear cells from peripheral blood and intestinal inflammatory cells might contribute to increased serum IL-6 levels. Recent evidence suggests that macrophages and colonic epithelial cells are the major cell types responsible for elevation of IL-6 in IBD (Kazuo *et al* 1995).

(4) IFN-γ: Cultured CD mucosal mononuclear cells spontaneously release IFN-γ, whereas blood mononuclear cells only release IFN-γ after stimulation (Fais *et al* 1991). Their findings indicate that in CD the intestinal lymphocytes are stimulated *in vivo* to produce IFN-γ and that the spontaneous IFN-γ production is compartmentalised to the gut lymphocytes.

2.4.3. Effects of cytokines on lipid metabolism

In vivo administration of TNF in rats rapidly increases plasma triglyceride concentrations by increasing the level of very low density lipoprotein of normal composition (Feingold *et al* 1987, Krauss *et al* 1990). In contrast to the findings in cultured fat cells, the effects of TNF on adipose tissue lipoprotein lipase were small when the triglyceride concentration was increased (Semb *et al* 1987, Grunfeld *et al* 1989b, Chajek-Shaul *et al* 1989). In addition, TNF had no effect on muscle lipoprotein lipase, whereas hepatic lipase and total post-heparin lipase levels were increased. Consequently, TNF did not slow the clearance of triglyceride-rich lipoproteins (Grunfeld *et al* 1991, Feingold *et al* 1990). The administration of IL-1 also rapidly increases plasma triglyceride concentrations without decreasing triglyceride clearance (Feingold *et al* 1991). Both TNF and IL-1 increase plasma triglycerides by stimulating hepatic lipogenesis and the production of very low density lipoprotein (Feingold *et al* 1991, Chajek-Shaul *et al* 1989). *In vivo* experiments in rats (Feingold *et al* 1987) have shown that TNF-α stimulates lipid synthesis by increasing hepatic lipogenesis. It does not stimulate lipid synthesis in other tissues including adipose tissue. The doses of TNF and IL-1 that stimulate the synthesis of hepatic fatty acids are similar to those that induce fever (endogenous pyrogen activity), suggesting that hepatic fatty acid synthesis will be increased during the normal response to infection (Adi *et al* 1992, Feingold *et al* 1989). IL-6 and IFN-α also increase hepatic lipogenesis (Feingold *et al* 1989, Grunfeld *et al* 1990a). Hepatic lipogenesis normally increases when excess

carbohydrate calories are consumed, so that energy can be stored more efficiently (calories of fat weigh less than calories of glycogen). However, such storage may be inappropriate during malnutrition, when the energy would be best used to maintain or restore muscle protein.

In addition to increasing hepatic synthesis of fatty acids, TNF rapidly mobilizes free fatty acids by stimulating peripheral lipolysis (Feingold *et al* 1990). The mobilized fatty acids are re-esterified into triglyceride in the liver and are re-secreted, contributing to the increase in very-low-density lipoproteins. Because very-low-density lipoproteins are cleared normally in animals given TNF, `futile cycling' results; fatty acids are shuttled from adipose tissue to liver and back to adipose tissue without being used for energy. Similar futile cycling has been described during infection (Wolfe *et al* 1985).

Daily injections of a constant dose of crude cachectin causes progressive weight loss in rodents (Cerami *et al* 1985). In contrast, rodents given purified recombinant TNF daily rapidly lose weight and then rapidly regain it (Patton *et al* 1987, Sochar *et al* 1988, Stovroff *et al* 1988). The acute weight loss is due to decreased intake of food and water accompanied by diuresis (Grunfeld *et al* 1989a, 1989b). However, rats given repeated treatment with TNF have persistent hypertriglyceridemia despite loss of anorectic or cachectic effects. The metabolic disturbances leading to hypertriglyceridemia thus do not inevitably cause wasting. Each metabolic disturbance must be placed in the context of total energy balance.

Mahony *et al* (1988) in an elegant study on pure strain NMRI mice model compared the weight loss produced by TNF with that produced by a restricted food and water intake (pair-fed controls), and by mitozolomide, a drug which in toxic doses induces weight loss with a similar decrease in nutrient and water intake. TNF produced a dose related weight reduction that was directly proportional to a decrease in both food and water intake. With chronic administration over a 5-day period the major weight loss was found to occur during the first 24-h after injection and thereafter the weight of treated mice increased toward that of controls. Acute administration of TNF produced hypoglycemia that was more severe than that observed with either mitozolomide or in pair-fed controls; there was also a reduction in the circulatory level of free fatty acids (FFA) and an increase in plasma triglycerides with TNF, while

mitozolomide and pair-feeding had no effect on the level of blood glucose or plasma triglycerides. Body composition analysis showed a loss of adipose tissue in TNF-injected and pair-fed animals after both acute and chronic treatment. Acute administration of TNF also induced a decrease in the total body water of treated animals which was similar to pair fed controls. The study showed that the weight loss produced by TNF arises from a combination of semi-starvation and a reduced water intake, and the effect only occurred with the first administration of TNF.

2.4.4. Cytokines in the induction of anorexia and cachexia

Since daily injections of TNF were unable to produce cachexia similar to that produced by crude cachectin, other experimental manipulations were tried. TNF can induce weight loss when given in progressively increasing doses, when infused continuously, or when secreted by tumours that are genetically engineered to produce TNF (Tracey *et al* 1988, Michie *et al* 1989, Oliff *et al* 1987, Teng *et al* 1991). In each of these models, however, TNF induces anorexia, and the weight loss or the negative nitrogen balance does not exceed that in controls given identical food.

Anorexia may contribute to wasting even in disorders in which metabolism is markedly disturbed. Colon cancers in rodents induce metabolic changes similar to those produced by TNF, such as increased hepatic synthesis of fatty acids, but not all colon tumours produce cachexia (Mulligan *et al* 1991). Animals that eat more to compensate for these metabolic disturbances maintain weight, whereas animals that do not eat more lose weight.

Although questions have been raised about the ability of TNF or IL-1 alone to promote negative nitrogen balance, the simultaneous infusion of the two cytokines leads to synergistic effects inducing hypermetabolism, glucose recycling, muscle catabolism and negative nitrogen balance (Tredget *et al* 1988, Flores *et al* 1989). Likewise the combination of IFN-alpha and TNF or IL-1 is synergistic in its effects on lipid metabolism (Grunfeld *et al* 1990b). In tumours that are engineered to produce TNF, there may be synergy between TNF and other tumour or host products, since tumours themselves render the host more susceptible to the toxic effects of exogenous TNF (Bartholeyns *et al* 1987). These studies suggest that synergistic

interactions between cytokines may be necessary for the wasting syndrome to develop.

A wide spectrum of metabolic disturbances can lead to wasting. Patients with overwhelming infection or burns have striking hypermetabolism and profound negative nitrogen balance (Brennan 1977, Wilmore *et al* 1974, Long *et al* 1979). In such patients, negative nitrogen balance and even weight loss may persist despite aggressive hyperalimentation (Brennan 1977). This situation can be contrasted with that associated with some forms of cancer in which there is no hypermetabolism (Dempsey *et al* 1984, Hansell *et al* 1986, Nixon *et al* 1988, Fredrix *et al* 1991). In yet other cancers and infections, metabolic disturbances range between these extremes. TNF-α has recently been implicated in the wasting in emphysema (Sridhar 1995) and in severe chronic heart failure (Levine *et al* 1990).

The processes that lead to hypermetabolism are not fully understood. Among them are futile or substrate cycling, the inappropriate use of substrates (such as the synthesizing a compound intended for long term storage when immediate energy use is needed), and the uncoupling of enzymatic events from energy formation. These processes all waste energy. They are usually found in the wasting syndromes but few data quantitatively link such disturbances to wasting.

In a study of serum levels of endogenous TNF in healthy people and patients with neoplastic or infectious diseases, only patients with visceral leishmaniasis and malaria were found to have a strikingly increased frequency of raised TNF levels. TNF may play a part in host defences against parasitic infections but it may also contribute to the striking wasting seen in these conditions (Scuderi *et al* 1986). Alveolar macrophages in tuberculosis produce TNF. The work of Lahdevirta *et al* (1988) in AIDS is of particular interest as it has suggested a correlation between TNF concentration and the severity of the disease. The question may be raised whether the wasting diathesis so commonly seen in AIDS is, at its root, attributable to unremitting production of TNF. There is little doubt that chronic TNF administration to rodents can by itself produce cachexia (Oliff *et al* 1987). Balkwill *et al* (1987) have reviewed the evidence for TNF production in cancer. Putative manifestations of TNF such as fever

may occur without producing a catabolic state thus once again reinforcing the dissociation between metabolic manifestations and the clinical features.

2.4.5. Effect of cytokines on bone metabolism

When leukocytes are exposed to mitogens or antigens in vitro, they release bone-resorbing activity into the culture supernatants which can be detected by bioassay (Horton *et al* 1972, Mundy *et al* 1984). Like many lymphocyte-monocyte product, this activity has been difficult to purify because of its low abundance in activated leukocyte cultures and the unwieldy bioassay required to detect biological activity. Partially purified preparations of this activity inhibit bone collagen synthesis in organ cultures of fetal rat calvariae (Raisz 1988). As both TNF-α and TNF-β are likely to be present in activated leukocyte supernatants, purified recombinant preparations were tested for their effects on bone resorption and bone collagen synthesis in vitro (Bertolini *et al* 1986). Both cytokines at 10^{-7}M to 10^{-9}M caused osteoclastic bone resorption and inhibited bone collagen synthesis. These data suggest that at least part of the bone-resorbing activity present in activated leukocyte culture supernatants may be due to these cytokines. IL-6 has similar bone-resorbing activity. Experimental colitis induced by instillation of trinitrobenzene-sulphonic acid with ethanol in a rat model results in bone loss (Fries *et al* 1994), providing further evidence of the role of intestinal inflammation in causing osteopenia.

A number of cytokines have been implicated in osteoclast formation. IL-1, TNF and IL-6 have all been shown to affect osteoclast development (Suda *et al* 1992). It has been recently shown that IL-11 derived from mesenchymal cells provides a more central signal for osteoclast formation (Girasole *et al* 1994). IL-6 increases the recruitment of osteoclast precursors to the osteoclast pool, but IL-6 may not play a role in osteoclast development in physiological conditions, and may only become important in oestrogen deficient states (Jilka *et al* 1992).

2.4.5.1. Bone resorption mediated by cytokines

Bone resorbing cytokines are generally active at low concentrations and IL-1 is the most potent (Gowen *et al* 1986a). These act primarily as stimulators of osteoclast

formation though they can affect mature osteoclast function as well (Thomson *et al* 1986). Both IL-1 and TNF-α stimulate multinucleated cell formation in long-term bone marrow culture (Pfeilschifter *et al* 1989), and these agents act as growth factors for osteoclast precursors, which are of the monocyte-macrophage lineage. Both GM-CSF and M-CSF stimulate multinucleated cell formation in long-term bone marrow cultures (MacDonald *et al* 1986). IL-6 has stimulatory effects on haemopoietic stem cell proliferation *in vitro* (Brandt *et al* 1990), and as such may increase the availability of osteoclast precursors. Some cytokines may have permissive effects on bone resorption by maintaining adequate numbers of early osteoclast precursors (GM-CSF, M-CSF and IL-6), whereas others act more locally and quickly to produce large numbers of the relatively more differentiated osteoclast precursors required for osteoclast formation (IL-1 and TNF-α). IFN-γ may be a candidate factor responsible for termination of osteoclast activity and inhibit basal and cytokine stimulated bone resorption (Gowen *et al* 1986b). IFN-γ decreases the number of osteoclasts and the recruitment of osteoclast precursors rather than having direct calcitonin-like inhibitory effects on mature osteoclasts (Klaushofer *et al* 1989). IL-4 has also been found to inhibit bone resorption both *in vitro* and *in vivo* (Watanabe *et al* 1990, Watanabe *et al* 1991).

2.4.5.2. Bone formation mediated by cytokines

Cytokines have effects on both the initial phase of recruitment and differentiation of osteoblast precursors and the later phase of bone matrix production and mineralisation by the mature osteoblasts. Two groups of cytokines with effects on bone formation can be identified. The first group contains those cytokines which stimulate bone cell proliferation but have inhibitory effects on mature osteoblast function. These agents are generally potent stimulators of bone resorption such as IL-1 and TNF-α. The second group can be categorized as members of the TGF-β family and includes TGF-β, IGFs and BMPs (Bone morphogenetic proteins). These agents stimulate both precursor proliferation and mature osteoblast function (Oreffo *et al* 1989).

2.4.5.3. Local production of cytokines in bone

Local production of cytokines within the bone microenvironment may be by non-skeletal haematopoietic or immunological cells, or by osteoblasts and osteoclasts. Osteoblasts are capable of secreting most of the osteotropic cytokines *in vitro.* Synthesis of local cytokines interacts with local cellular events (osteoclast activation or initiation of bone formation) and the effect depends on the stage of development of the skeleton (foetal versus adult) (MacDonald *et al* 1992).

2.4.5.4. Regulation of cytokine actions in bone

IL-1 has synergistic effects on bone resorption *in vitro* with TNF-α (Gowen 1988) and IL-6 (Black *et al* 1990). IL-1 also induces the synthesis and secretion of TNF-α (Gowen *et al* 1990). TGF-β transcription however is regulated by osteotropic hormones rather than cytokines and levels are reduced in vitamin D deficient animals (Finkelman *et al* 1991). Control mechanisms include inhibitory effects of soluble receptors or receptor antagonist proteins such as IL-1 receptor antagonist. Bone metabolism is influenced by the combined effects of a number of cytokines and a high degree of redundancy may exist with no cytokine having a unique role. Interaction with the extracellular matrix or modulation of integrin expression may provide further complicated regulation.

2.4.5.5. The role of cytokines in the pathogenesis of bone disease

Bone destruction in multiple myeloma, rheumatoid arthritis and post-menopausal osteoporosis may result from excess local or generalised production of cytokines. The malignant plasma cells of myeloma release cytokines such as IL-6 (Kawano *et al* 1988), TNF-α (Garrett *et al* 1987) and IL-1 (Kawano *et al* 1989) mediating osteoclast activity and bone destruction. Cytokine levels in the rheumatoid joint favour osteoclast action and juxta-articular osteoporosis and bony erosions. These include high levels of IL-1 (Nouri *et al* 1984), TNF-α (Saxne *et al* 1988), IL-6 (Houssiau *et al* 1988) and M-CSF (Firestein *et al* 1988), but low levels of IFN-γ (Firestein *et al* 1987). In post-menopausal osteoporosis, evidence supports the indirect effect of oestrogen via modulation of cytokine production. Both IL-1 and TNF secretion

by peripheral blood monocytes are raised in post-menopausal women (Pacifici *et al* 1987, Ralston *et al* 1990), and the administration of oestrogen to oophorectomized women is associated with a normalisation of cytokine production (Pacifici *et al* 1991). Osteoblast IL-6 production may also be modulated by oestrogen (Girasole *et al* 1990).

2.5. Body compartments: Lean Vs fat compartments

Most body composition methods are based upon the model in which the body consists of two chemically distinct compartments, fat and lean or fat-free (Keys *et al* 1953, Lukaski 1987). The chemical composition of lean is assumed to be relatively constant with a density of 1.1g/mL at 37°C (Keys *et al* 1953, Brozek *et al* 1963, Behnke *et al* 1942), a water content of 72-74% (Pace *et al* 1945), and a potassium content of 60-70 mmol/kg in men and 50-60 mmol/kg in women (Boddy *et al* 1973). Fat, or stored triglyceride, which is anhydrous and potassium free, has a density of 0.900g/mL at 37°C (Mendez *et al* 1960a, Mendez *et al* 1960b). Any mixture of fat and lean will result in an average density somewhere between 1.10 and 0.90. A variety of methods to measure human body composition indirectly are available, but few can be adapted for routine use at the bedside.

2.5.1. Methods of assessment of body composition

For the sake of convenience, I have divided these methods into simple methods, which do not need complicated expensive equipment and sophisticated methods which need equipment generally available only in Medical Physics departments of tertiary care institutions.

2.5.1.1. Simple methods

(1) Anthropometry: This is currently the most widely used method clinically for measurement of body composition. It depends on the fact that most of the fat stored in the body lies immediately under the skin (Edwards 1950) and the thickness of a fold of skin picked up indicates the amount of subcutaneous fat. The measurement of skinfold requires skill and training (Ruiz *et al* 1971) and it is important to measure triceps skinfold at exactly the correct site, otherwise false results are obtained. A

theoretical limitation to the skinfold measurement is that it assumes a constant relationship between subcutaneous and deep fat stores, which was not confirmed by measurement at postmortem examinations (Alexander 1964). A single skilled, trained observer may obtain reproducible results with an error of about 3% of body weight (i.e. 2 kg of fat in an average subject), but results will be very variable if unskilled, untrained observers make the measurements. The limitations of anthropometric methods have been highlighted in a review (Lukaski 1987). Hence, the method is not suitable for use by nursing staff in a busy outpatients' clinic.

(2) Densitometry: The method assumes that the body is composed of two distinct compartments, fat and lean, and that it is possible to determine each of these compartments from the measured whole-body density. Underwater weighing (Akers *et al* 1969) is widely used to determine body volume - though theoretically simple, it requires cumbersome, expensive equipment and is only suitable for fit, healthy subjects. Other instruments to measure body volume, such as body volumeter (Garn *et al* 1963) and plethysmograph (Garrow *et al* 1979) suffer from the same limitations and are not suitable for routine clinical application.

(3) Quetelets's index: The use of Quetelet's index (Weight in kg/Height in metre^2) as a measure of fatness has been analysed by Garrow *et al* (1985). Though a useful measure in obesity, its value in under-nourished states has not been rigorously evaluated. In this thesis, I have used Quetelet's index to compare other methods of measurement of body composition.

(4) Urinary creatinine excretion: Although many tissues take up creatine, the preponderance (98%) of it is located in the skeletal muscle, mostly in the form of creatine phosphate (Borsook *et al* 1947). Creatinine is formed by non-enzymatic hydrolysis of free creatine liberated during the dephosphorylation of creatine phosphate. It has generally been accepted that urinary creatinine excretion is related to lean mass and muscle mass (Boileau *et al* 1972, Forbes *et al* 1976). Large individual variability in daily urinary creatinine excretion (Greenblatt *et al* 1976, Ransil *et al* 1977), influence of dietary creatine intake (Crim *et al* 1975) and the need for accurately timed urine collections make urinary creatinine excretion a generally unreliable method of measurement of body composition.

2.5.1.2. Sophisticated methods

(1) Measurement of total body water: Water occupies a relatively fixed fraction (73.2%) of the lean mass and is not present in fat compartment (Pace *et al* 1945). Hence, lean mass is equal to total body water divided by 0.73. Investigators have used the isotopes of hydrogen, deuterium and tritium, to quantitate body water volumes by isotope dilution in healthy and diseased individuals (Lukaski 1987). Implementation of this technique is difficulty outside of a specialized research laboratory.

(2) Measurement of total body potassium: Potassium is essentially an intracellular cation that is not present in stored fat. Potassium is labelled with the natural radioactive isotope, ^{40}K, so that each gram of potassium emits about three gamma rays of high energy (1.46 MeV) each second, which can be detected by suitable apparatus and the total body potassium can be estimated (Boddy *et al* 1976, Smith *et al* 1979). Quantitation of total body potassium requires specially constructed counters that consist of a large shielded room (to reduce background radiation from cosmic and terrestrial sources) containing a gamma ray detection system connected to a suitable recording device. The cost of such determinations, including instrumentation and technical support, may be prohibitive for clinical application.

(3) Neutron activation analysis: The development of *in-vivo* total body neutron activation analysis has provided a reliable technique for measurement of the absolute content of sodium, chloride, phosphorus and nitrogen (Vartsky *et al* 1979, Vartsky *et al* 1984). Facilities for this investigation are available in only a few specialist centres and the high cost, need for skilled operators, lack of portability and the use of ionizing radiation preclude wide application of this technique.

(4) Absorptiometry: The ability of bone mineral to absorb energy from a photon beam of given energy is known. The mass of bone mineral in a limb can be estimated by scanning across a limb with a beam of photons of known energy and observing the energy which emerges at the other side (Mazess 1971, Christiansen *et al* 1977). With single-photon absorptiometry, the bone must be enclosed in a constant thickness of soft tissue and water baths, tissue-equivalent substances and local compression have been used as control materials for this purpose. It is not feasible to scan the total body with the single-photon technique. Also, the use of single-photon absorptiometry

measurements of the appendicular skeleton may not be as sensitive a predictor of osteoporosis as are determinations, made by dual- photon absorptiometry of the spine, a prime target of demineralisation. This method is not used for total body composition analysis.

Total body bone mineral content and lean body mass are measurable using dual-photon absorptiometry (DPA), which eliminates the need for constant soft-tissue thickness across the scan path. This method uses a whole-body rectilinear scanner and a high activity source of gadolinium-153 which emits energy at two discrete peaks (44 and 100 keV). Body composition measured by dual-photon absorptiometry and densitometry are well correlated (Mazess *et al* 1984).

Dual energy X-ray absorptiometry (DEXA) is an accurate and reproducible method for measuring lean body mass and agrees well with that measured by prompt neutron activation analysis and by total body water methods (Hannan *et al* 1993). I have used this technique to validate bioelectrical impedance analysis results and also relate bone mineral density to lean body mass in IBD patients. Details are given in chapters VII and VIII. All absorptiometric techniques need trained personnel and expensive equipments.

(5) Computerised tomography (CT): This has provided valuable information about the relative proportions of fat which are subcutaneous or intra-abdominal (Kvist *et al* 1988) and highlighted limitations of anthropometry based only on subcutaneous fat. Practical constraints limit the general use of CT scanners for body composition analysis. The examination takes quite a long time, significant radiation doses are involved and the cost and general availability of modern CT scanners prevent the routine use of this technique for only body composition assessment.

(6) Magnetic resonance imaging (MRI): MRI does not involve ionising radiation and can generate images in response to intrinsic tissue variables. In addition to hydrogen, MRI can image phosphorus and future prospects include carbon, nitrogen, sodium and chlorine. It can quantitate total fat mass and discriminate differences in regional fat distribution (Mallard 1986). High cost, restricted availability and technical problems with spatial resolution make this technique investigational at present.

(7) Electrical conductivity: Two methods have been developed which depend on the difference in electrical conductivity of lean tissue (which is virtually an electrolyte solution, and a good conductor) and fat, which is a non-conductor.

The first method, bioelectrical impedance analysis (Lukaski *et al* 1986, Lukaski 1987) uses a pair of electrodes attached to the left hand and left foot of the subject. A current of 800 μA at a frequency of 50 MHz is passed between the outer electrodes, and the voltage drop is measured at the proximal electrodes, from which the resistance (strictly impedance, since it is an alternating current) of the tissues is calculated. The measured impedance is entered into a regression equation, together with anthropometric data such as weight, height, age and gender. The coefficient of variation of this method, determined in 14 men in whom impedance was measured on five consecutive days, was <2% (Lukaski *et al* 1985). Comparison of densitometrically determined and impedance predicted lean body mass yielded an error of prediction of 2-2.5 kg and a calculated error of relative body fatness of 2.7% (Lukaski *et al* 1986). Relative to densitometry the observed error of calculated body fatness was larger by anthropometry (3.9%) than by impedance (2.7%). User-friendly bioelectrical analysis machines (Smye *et al* 1993, Fuller *et al* 1994) offered a technique that could readily be used by untrained staff in busy outpatient clinic settings. I evaluated one of these machines in IBD patients (chapter VII).

In total body electrical conductivity (TOBEC) system the subject lies within a solenoid coil through which radiofrequency pulses are fed at a frequency of 5 MHz (Segal *et al* 1985). This generates an alternating magnetic field within the coil which induces a response in the subject depending on the conductivity of the tissues, and the strength of the evoked field is measured by a secondary coil. The advantage of this system is that it is very quick; the disadvantage is that the evoked field depends on the shape of the subject in addition to his fat content. Second generation equipments are more reliable, but TOBEC is much more expensive than bioelectrical impedance analysis.

(8) Other methods: Infrared interactance, endogenous urinary 3-methylhistidine excretion, plasma creatinine and ultrasonography have been used to analyse body

compartments; these are not in common use and have been reviewed elsewhere in details (Lukaski 1987).

2.6. Pathophysiology of bone loss

2.6.1. Types of bone

There are two types of bone tissue in adults, cortical or compact bone and spongy or cancellous bone. Eighty percent of the skeletal mass is cortical. The remaining cancellous bone comprises trabecular plates and rods of tissue which interconnect with each other (Kanis 1994). The disposition of trabecular plates and rods is oriented predominantly according to lines of stress.

2.6.2. Matrix and mineral

Bone comprises an organic matrix, a mineral phase and bone cells. The majority of the matrix is composed of collagen. Adult bones contain type 1 collagen laid down by osteoblasts. Each unit of collagen (tropocollagen) comprises a protein heterotrimer consisting of two α-1 chains and one α-2 chain. Post-translational modification of synthesised protein consists of hydroxylation of proline and lysine. Hydroxyproline and hydroxylysine release during the metabolism of bone can be utilized as an index of bone breakdown. Glycosylation and the formation of cross-links with other tropocollagen macromolecules permit their assembly to form collagen fibrils and evidence of such activity can be measured in serum and urine. Other proteins incorporated within the collagen matrix during or after its formation include proteoglycans, glycoproteins, osteocalcin and osteonectin. Osteocalcin is used to assess the rate of bone turnover in the evaluation of osteoporosis. Hydroxyapatite crystals composed of calcium, phosphate and carbonate form the mineral phase of bone. The crystals also contain other ions such as sodium, magnesium and fluoride.

2.6.3. Bone cells

All bone surfaces are covered by cells with distinct morphological and functional features (Kanis 1994). These include osteoblasts, osteoclasts and osteocytes. The osteoblast is the cell responsible for the synthesis of collagen and

other bone proteins. Osteocytes are osteoblasts which have been trapped within the bone matrix during the process of bone formation. The osteoclast is a multinucleated cell which is responsible for bone resorption. It degrades fully mineralized bone by attaching onto a bone surface and secreting acids and lysosomal enzymes.

2.6.4. Bone loss

Bone remodelling is a constant process in adult bone. Old bone is removed by osteoclasts and replaced by new bone formed by osteoblasts. The term coupling refers to the sequence of bone resorption followed by formation, and the term balance describes the equality of these two processes within individual remodelling units (Compston 1995b). Mechanical stimuli and endocrine factors such as sex hormones, glucocorticoids, thyroxine, growth hormone and parathyroid hormone exert important effects on bone cells. These effects may be mediated through prostaglandins, nitric oxide, cytokines and growth factors (Russell 1989, Lanyon 1992, MacDonald *et al* 1992). There are two mechanisms of bone loss in osteoporosis:- (1) Increased bone turnover where the number of remodelling units is increased. This results in an increase in the percentage of bone surface occupied by resorption cavities. (2) Negative remodelling imbalance where the bone loss results from bone formation being less than resorption within a remodelling unit.

Increased bone turnover are associated with an increased likelihood of trabecular penetration and erosion, with loss of connectivity of the cancellous bone structure. Remodelling imbalance is associated with trabecular thinning and relatively greater preservation of bone architecture.

2.6.5. Corticosteroids and bone loss

Corticosteroids affect both bone resorption and formation. Resorption is mediated partly by increased parathyroid hormone secretion secondary to calcium malabsorption and increased renal losses. Corticosteroids directly affect osteoblast proliferation, differentiation and activity, and also inhibit bone formation by reduced local production of prostaglandin E_2 and insulinlike growth factor 1. Reduced production of gonadal hormones by the adrenal gland, ovaries and testes may

contribute to corticosteroid -induced bone loss (Compston 1995b). Cancellous bone is metabolically more active than cortical bone and is preferentially affected in corticosteroid induced bone loss. Thus, the spine and ribs are especially vulnerable. Histomorphometric studies in patients with corticosteroid-induced osteopenia have shown low or normal bone turnover with negative remodelling imbalance due to reduced bone formation (Bressot *et al* 1979). Calcium kinetic studies suggest that increased bone turnover may occur during the early stages of treatment (Cannigia *et al* 1981).

2.6.6. Physical activity and bone mass

Epidemiological data suggest that lack of physical activity is a risk factor for osteoporotic fracture.

2.7. Measurement of bone mineral density

Osteoporosis is characterised by low bone mass and disruption of bone architecture, leading to reduced bone strength and increased fracture risk. Measurement of bone mineral density (BMD) provides the best prediction of future fracture risk (Wasnich *et al* 1985, Hui *et al* 1988, Cummings *et al* 1993). DEXA is the most accurate and reproducible method of measuring BMD (Compston *et al* 1995a). It involves a low radiation dose and can measure bone mass in both axial and appendicular skeleton. DPA, single photon absorptiometry, single X-ray absorptiometry, broadband ultrasonic attenuation and quantitative CT scanning are also accurate, alternative methods of measuring BMD (Compston 1995b). Single photon absorptiometry and single X-ray absorptiometry are suited for BMD measurement at the radius, whereas broadband ultrasonic attenuation is suited for the os calcis. Axial bone mass may be determined by DEXA, DPA and quantitative CT. Prospective studies have demonstrated an increasing fracture risk with decreasing BMD. A reduction of 1 SD in BMD is associated with a two- to threefold increase in fracture risk (Wasnich *et al* 1985, Hui *et al* 1988, Cummings *et al* 1993).

Lateral radiograph of the dorsal and lumbar spine provide evidence of subtle changes in vertebral shape. Bone biopsy should be considered in severe osteopenia to exclude osteomalacia.

2.7.1. Osteopenia in IBD

Vitamin D deficiency in CD and associated bone disease has been described (Driscoll *et al* 1982), though osteopenia with normal vitamin D metabolites (Hessov *et al* 1984) is more common. Genant *et al* (1976) using spinal X-rays and measurements of metacarpal metacarpal cortical thickness and bone mineral content, found a significantly reduced bone mass in adolescents and adults with IBD. A number of studies established the high prevalence of osteoporosis in IBD, both CD and UC (Compston *et al* 1987, Motley *et al* 1988, Pigot *et al* 1992). Decrease in total body calcium has been described (Ryde *et al* 1991) and histomorphometric data are consistent with reduced bone turnover (Croucher *et al* 1993). These aspects are discussed in details in subsequent chapters and will not be repeated here.

2.8. Characterisation and stratification of IBD: review of predecessors' work on whole gut lavage fluid

In order to study the gut immune system and inflammation in IBD, a range of components should be assessed. With very few exceptions, tests on components of the systemic immune system are virtually useless as indices of mucosal immunity at gut level. Some general information on the function of the mucosa-associated lymphoid tissues can be obtained from studies of saliva or tears, but these materials cannot provide organ-specific information relevant to the gut.

The technique of whole gut lavage with non-absorbable polyethylene-glycol-based solution has been widely applied in clinical practice for cleansing of the bowel prior to barium enema, colonoscopy or colonic surgery. After gut cleansing is complete, the clear fluid passed per rectum, whole gut lavage fluid (WGLF) is essentially a whole gut perfusate. Our studies in adults and children have shown that

this material can be used for biochemical and immunochemical assays to assess intestinal immunity, inflammation and gut losses of protein and blood.

2.8.1. Gastrointestinal protein loss - index of activity in IBD

Fluid obtained by whole gut lavage normally contains traces of IgG, albumin and α_1 - antitrypsin. Normal values, based on results for 63 immunologically normal patients or volunteers are: IgG (by ELISA) 1-10 μg/mL; albumin (by immunoturbidimetry) 1-26 μg/mL; α_1 - antitrypsin (by immunoturbidimetry) 1-19 μg/mL (Choudari *et al* 1993). My predecessors' work has shown that higher concentration of these proteins were found in WGLF from patients with IBD (O'Mahony *et al* 1990, Brydon *et al* 1993). A prospective study of gut lavage in IBD patients (Choudari *et al* 1993) in whom disease activity was simultaneously assessed by using the Crohn's Disease Activity Index (CDAI) (Best *et al* 1976) or the Powell Tuck Index (PTI) (Powell-Tuck *et al* 1978). For IgG, concentrations in lavage fluid correlated closely with activity indices. In CD, $r = 0.723$ ($p<0.0001$), in UC, $r = 0.714$ ($p<0.0001$) for CDAI or PTI plotted against WGLF IgG. Results for WGLF albumin and α_1 - antitrypsin concentrations were generally similar to those for IgG, but less sensitive in detecting active disease.

When serial samples were collected after the first clear specimen, no significant differences in the concentrations of these proteins could be detected between the serial samples (Sallam *et al* , submitted) - these data established WGLF as a gut perfusion system. WGLF analysis also showed that data based on analysis of faeces may be highly misleading (Ferguson *et al* 1995).

2.8.2. Measurement of gastrointestinal blood loss

Occult gastrointestinal bleeding can be measured by using a highly sensitive technique, Hemoquant, for assay of haemoglobin in WGLF (Brydon *et al* 1992). In patients with a normal gastrointestinal tract, WGLF haemoglobin concentration ranged from 0.5 - 5.1 μg/mL equating to an estimated daily occult blood loss of 0.1 -1.2 mL. High values were found not only in patients with colorectal cancer, but also in active IBD.

2.9. Neutrophil migration into the gut lumen in IBD

Neutrophils are the predominant effector cells within the active lesions of IBD and probably mediate much of the tissue damage. Indium^{111} - labelled leukocyte studies have demonstrated rapid accumulation of radiolabelled cells in the inflamed intestine, followed by migration into the crypts and thence into the intestinal lumen (Saverymuttu *et al* 1983a,b,c, 1986a). These studies showed that monitoring neutrophil migration could be of potential value in the clinical assessment of IBD patients. The introduction of ^{99m}Tc - HMPAO as a leukocyte label further refined the technique and provided much better scans (Scholmerich *et al* 1988).

There are several groups of molecules which may be acting as neutrophil chemoattractants in IBD. These include complement, eicosanoid and cytokine families and proinflammatory bacterial peptides. Recent studies have focused attention on IL-8, a non-glycosylated single peptide chain which mediates activation and chemotactic attraction of neutrophils (Baggiolini *et al* 1989). The human neutrophil expresses at least two distinct receptors, high and low affinity, for IL-8 (Holmes *et al* 1991, Murphy *et al* 1991). This cytokine was originally isolated from endotoxin-treated monocytes, but a variety of other cells may be induced to produce IL-8, and stimuli include IL-1 and TNF-α. Evidence that IL-8 may be implicated in the local tissue lesion of IBD comes from rectal and sigmoid perfusion studies, which show that myeloperoxidase released from neutrophil granules correlates with IL-8 in the perfusate in UC (Raab *et al* 1993).

Mitsuyama *et al* (1994) demonstrated that raised IL-8 content of affected colonic mucosa in active IBD correlates with neutrophil counts in the mucosa. This is strong evidence of a central role for IL-8 in the pathogenesis of neutrophil infiltration. These workers also observed that mucosal IL-8 levels in macroscopically unaffected areas of the colon were similar to those in colonic biopsies from immunologically normal control patients. Measurement of serum IL-8 concentration was unhelpful.

An important unanswered question is the source of IL-8 in IBD. Mucosal monocytes and macrophages are known to produce IL-8, and IL-8 is also expressed by human epithelial cell lines (Eckmann *et al* 1993). There may be bi-directional

communication between intestinal epithelial cells and mucosal immune cells, whereby IL-1 and TNF-α released by activated macrophages may result in increased IL-8 secretion by intestinal epithelial cells. However, caution is needed in extrapolating data from *in vitro* studies - epithelial cell lines also express IL-1 and yet immunohistochemical and *in situ* hybridization studies have shown that lamina propria mononuclear cells and not epithelial cells are the source of IL-1 in intestinal mucosal biopsies (Youngman *et al* 1993).

One potential mechanism of action of IL-8 is via regulation of expression of leukocyte adhesion molecules. Binding of neutrophils to the vascular endothelial cells may be mediated through well characterized adhesion molecules such as CD11a/CD18 complex (LFA-1) which binds to CD54 (ICAM-1). In active IBD, the expression of CD54 is strikingly increased (Malizia *et al* 1991). Another family of adhesion molecules, the LEC-CAM, present on neutrophils and endothelium, mediate adhesion by a lectin domain. This promises to be one of the central areas for future research and therapeutic intervention.

Some of the traditionally investigated chemoattractants such as leucotriene-B_4 may be a secondary consequence of neutrophil infiltration, while other mediators such as those of the complement family (C3b, C5a) may be important in the final pathway of tissue injury by perpetuating the inflammation. Though several groups have investigated the role of platelet-activating factor (PAF) in IBD, no correlation between degree of tissue inflammation or disease activity and PAF has been reported. An important area that needs further experimental clarification is the relationship of neutrophil chemoattractants of bacterial origin such as formyl-methinyl-leucyl-phenylalanine (FMLP) and production of tissue mediators such as IL-8.

2.10. Role of inflammatory mediators in wound healing and stricture formation in the intestine

Intestinal smooth muscle cell has the capacity to respond to inflammation by proliferating and synthesizing collagen. The inflammatory cytokines involved in this response include transforming growth factor-β (TGF-β), IL-1, TNF-α, insulinlike growth factor -1 (IGF-1), fibroblast growth factor (FGF), platelet derived growth factor (PDGF),

prostaglandins and leukotrienes (Graham 1994). It is probable that under certain circumstances, the smooth muscle cells are synthesizing these cytokines themselves (Raines *et al* 1989).

TGF-β_1 is a homodimeric polypeptide involved in the regulation of growth and differentiation of both normal and transformed cells. It stimulates the synthesis of extracellular matrix components and inhibits matrix degradation, resulting in the formation of fibrosis and tissue repair (Moses *et al* 1990, Ignotz *et al* 1986). It is supposed to be the mediator of fibrosis in many disease processes, such as liver cirrhosis (Castilla *et al* 1991), glomerulonephritis (Okuda *et al* 1990), or idiopathic pulmonary fibrosis (Broekelmann *et al* 1991). Evaluation of the expression of TGF-β in tissues affected by IBD has not yet been studied in a comprehensive manner. TGF-β exhibits a variety of effects which may play a role in IBD (Podolsky 1994). Initial studies demonstrate high levels of TGF-β expression, both mRNA and bioactive peptide, in actively inflamed IBD tissues relative to normal tissues (Rossiter *et al* 1990). The enhanced expression of TGF-β is similar in mucosa involved by either UC or CD.

Insulinlike growth factor I and II (IGF-1 and II) are produced by intestinal epithelial cells and model cell lines (Brown *et al* 1986, Laburthe *et al* 1988, Jaffe *et al* 1990). These proteins facilitate proliferation of epithelial cells and may also have effects on other cell populations within the inflamed intestine as well. As yet, the expression and effects of IGF in IBD have not been examined. Many cell types, including neoplastic cells, are capable of synthesizing IGFs and responding to IGFs locally. Thus, the physiologically relevant IGF levels include both the tissue and the circulating levels. IGFs have mitogenic activity, but they also promote functions in differentiated cells. Soluble specific binding proteins (BP s) are present in blood, extracellular fluids and cell culture media and can inhibit or augment the actions of IGFs at the membrane receptor level. IGF-1 is a potent mitogen for fibroblasts (Conover *et al* 1989) and smooth muscle cells (Elgin *et al* 1987) and induces collagen synthesis *in vitro* (Hock *et al* 1988, Goldstein *et al* 1989). IGF-1 appears to play an important role in tissue remodelling and repair (Mueller *et al* 1991). Emerging evidence

suggests that proinflammatory cytokines such as IL-1 may induce IGF-1 *in vitro,* linking IGF-1 to key mediators of the inflammatory response in IBD (Kirstein *et al* 1992).

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SECTION II

Nutritional studies in inflammatory bowel disease

Chapter III

METHODS OF INVESTIGATING BONE MINERAL DENSITY AND BODY COMPOSITION

I have described below the method used for measuring bone mineral density (BMD), the sites chosen for testing and the confounding variables that were considered. The methods used for measuring body composition are also described. These include established methods to measure lean body mass in a Medical Physics Department as well as use of a newly available machine that can be used at the bedside by personnel with no specialised training.

3.1. Measurement of bone density - consideration of the confounding variables

BMD is affected by a number of variables (Stevenson 1989). The following variables were recorded in a questionnaire administered to each patient by me.

3.1.1. Physical activity: validation of questionnaire

The level of physical activity at the time of diagnosis and the pre-morbid level of physical activity when the patient was last free from IBD manifestations were recorded on a 1-5 scale as follows:

1. Predominantly homebound.
2. Walks once a week for shopping.
3. Average activity; no formal exercise or hard work.
4. Active: gymnasium, games, formal exercise at least once per week.
5. Regular athletic activities.

The level of physical activity at the time of follow-up measurement of bone density was also recorded.

The scale of physical activity was validated against the Baecke habitual physical activity questionnaire, a reliable self-administered measure of physical activity (Baecke *et al* 1982). Seven study patients, 10 IBD patients not in the study and 10 healthy volunteers completed the Baecke habitual physical activity questionnaire and were also graded on the scale given above. Age range of the

participants was 18-57 years (16 male; 11 female). The Baecke questionnaire scores three dimensions of physical activity, namely, (a) work, (b) sport, (c) leisure-time. Grades 1 and 2, grade 3 and grades 4 and 5 significantly differed from each other in the mean scores of all the three dimensions. Grade 1 significantly differed from grade 2 only in `work' score and grade 4 differed from grade 5 only in `sport' score.

3.1.2. Smoking

Smokers have lower bone mineral density than non-smokers (Daniell 1976, Stevenson *et al* 1989). All patients were categorized as smokers, non-smokers or ex-smokers. The duration of smoking and the average number of cigarettes smoked per day were also recorded.

3.1.3. Menstrual history

Secondary amenorrhoea and postmenopausal oestrogen deficiency are well known risk factors for osteoporosis (Richelsen *et al* 1984, Stevenson *et al* 1982, Stevenson *et al* 1989). All patients were questioned regarding their menstrual history, intake of oral contraceptive pill and hormone replacement therapy. They were also asked whether they had been commenced on hormone replacement therapy because of osteoporosis.

3.1.4. Developmental stage in children: bone age

All patients aged less than 20 years had bone age assessed from a radiograph of the left wrist and hand (Greulich and Pyle). All radiographs were reported by a single, experienced radiologist.

3.1.5. Alcohol

Alcohol abuse is associated with decreased bone mineral density though this effect has been demonstrated only in heavy drinkers (>40 units per week for at least 2 years) (Feitelberg *et al* 1987, Stevenson 1988). A history of alcohol intake was recorded from all patients.

3.1.6. Drugs

Drugs which may affect bone density such as thyroxine, oestrogen and corticosteroids were recorded. In case of corticosteroids, a detailed record of doses and duration were kept so that cumulative corticosteroid use could be calculated.

3.1.7. Other diseases

None of the patients was pregnant and none suffered from known cholestasis, renal disease, hypogonadism, previous gastric surgery or thyroid or parathyroid disorders.

3.2. Measurement of bone density

3.2.1. The machine and setting

A Hologic QDR-1000/W (Hologic Inc, Waltham, MA, USA) scanner was used to obtain dual energy X-ray absorptiometry (DEXA) measurements of BMD. The principle underlying this method has been reviewed in chapter II. Figure 3.1 shows the DEXA scanner in the Medical Physics department of the Western General Hospital. Figure 3.2 and 3.3 show typical bone scans obtained from the spine and forearm respectively.

3.2.2.Reproducibility

For scan-rescan measurements at our centre, the coefficient of variation for the lumbar vertebrae is 0.8% and that for the forearm is 0.5%. The details of the reproducibility of Hologic QDR-1000W scans are given below:

Spine BMC

Intra-observer = 0.73%

Inter-observer = 0.82%

Coefficient of variation for repeat measurements (CoV) = 1.59%

Spine BMD

Intra-observer = 0.36%

Inter-observer = 0.27%

CoV = 0.88%

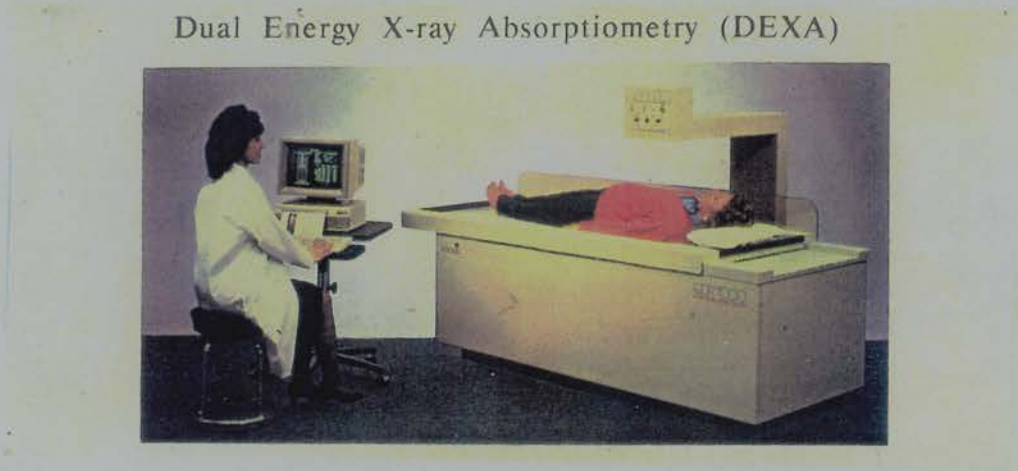


Figure 3.1. Hologic QDR-1000/W DEXA scanner.

Forearm BMC

Mid Distal CoV = 1.91%

Ultra Distal CoV = 1.09%

Total CoV = 1.40%

Forearm BMD

Mid Distal CoV = 0.65%

Ultra Distal CoV = 0.85%

Total CoV = 0.53%

3.2.3. The sites of measurement

The two sites of measurement chosen were lumbar L1-L4 vertebrae (Newton *et al* 1993) and of right forearm (both radius and ulna). These two sites have the best scan-rescan reproducibility and the Medical Physics department has extensive experience in these scanning at these two sites. In our centre, measurement of BMD at the femoral neck has quite a low test-retest reliability. For scan-rescan measurements the coefficient of variation for the lumbar vertebrae was 0.8%, whereas that for the femoral neck was 2% (Newton *et al* 1993). The lumbar vertebrae also contain 50% trabecular bone which is more metabolically active than cortical bone found in the femoral neck and is therefore, theoretically more likely to show early bone density changes. Total body bone density has a large coefficient of variation, often depends on precise positioning of the body and it has not been shown that whole body DEXA would give additional information regarding BMD. It was however used for body composition data. The forearm sites chosen had a narrow coefficient of variation, and measured a combination of trabecular and cortical bone as mentioned below. The bone mineral content (BMC) divided by the area of the bone gave the BMD in grams/cm^2. The average BMD values for L1-L4 and for the 3 forearm sites were used for calculations. The Hologic forearm application protocol defines a global region of interest encompassing 3 individual forearm sites which are: i) the 1/3 distal region is defined as a region 20mm wide centered at a distance equal to 1/3 of the forearm length measured from the distal tip of the ulna and contains mostly cortical bone; ii) the ultra distal region is 15mm in length positioned proximal to the end plate of the radius. This region excludes the end plate of the radius and contains mostly

trabecular bone; iii) the mid distal region is the region between the 1/3 and ultra distal regions and contains both trabecular and cortical bones. At our centre, dominant and non-dominant forearms were found to be comparable.

3.2.4. Controls

Normal values for BMD readings with the Hologic QDR-1000/W scanner were available as mean BMD (±SD). The normal values for L1-L4 were available from age 0-85 for both males and females; for forearm measurements normal values were available from age 20-85 for both males and females. The normal values were provided by Hologic reference database. The manufacturers supplied data obtained from healthy Caucasian volunteers of either sex (spine: 605 female, 294 male subjects; forearm: 366 female, 70 male subjects). None were on steroids, anticonvulsants, fluoride, diuretics or oestrogens. Subjects aged over 60 years had a lateral spine radiograph and if there was evidence of vertebral fractures or spine BMD below 0.82g/cm^2, subjects were excluded. Statistical thresholds were set and individuals below the first and above the 99th percentiles were excluded. In order to ensure that the Hologic reference database (based on an U.S. population) was satisfactory in the UK, 100 healthy normal volunteers from the local Scottish population were recruited. BMD values obtained from locally recruited control subjects were not significantly different from the Hologic reference database and hence the manufacturers' database was used for our control values.

3.2.5. Typical scans

Figure 3.4 and 3.5 illustrate a spine and a forearm scan from the same patient with marked osteopenia. The BMC divided by the area of the bone gives the BMD. This can then be plotted on a nomogram derived from normal subjects as shown in the figure or a Z-score may be derived as discussed below.

3.2.6. Analysis of results using the Z-score

Z-score is a standard statistical method (Harper 1982) of assessing the deviation of a measurement from the mean of a normal population when the standard deviation of the normal population is known. Z scores were calculated for each patient using the formula:

$$\frac{\text{Measured BMD - Normal BMD(age and sex matched)}}{\text{Standard Deviation of the normal BMD}}$$

Z scores were available for all BMDs from L1-L4; Z scores for forearm could only be calculated for patients aged 20 or over.

3.3. Biochemical assessment of Ca^{++} homeostasis

All these investigations were available as routine assays in the clinical chemistry department of the Western General Hospital.

3.3.1. Routine serum and urinary analysis

1) Plasma calcium and urinary calcium/creatinine ratio measured in a 24-hr sample of urine collected in a special container containing HCl, 2) Plasma phosphate and urinary phosphate/creatinine ratio measured in a 24-hr sample of urine. 3) Plasma albumin. 4) Plasma alkaline phosphatase. 5) Plasma 25-hydroxyvitamin D 6) Plasma immunoreactive parathyroid hormone (iPTH) 7) Urinary hydroxyproline/creatinine ratio measured in a 24-hr sample of urine collected in a special container with toluene.

Blood samples were taken after an overnight fast in order to avoid any influence of meals on serum phosphate concentration. Plasma total calcium, phosphate, alkaline phosphatase and urinary calcium, phosphate creatinine concentrations were measured by standard routine methods (Kodak, Ektachem E700C autoanalyser, USA). Urinary hydroxyproline was measured by colorimetric method (Varley 1980).

3.3.2. 25-OH-vitamin D

25-OH-D levels were measured in 4 patients in winter, 8 in spring, 8 in summer and 10 in autumn, and compared with seasonally adjusted reference values. Plasma 25-OH-Vit D (D_2+D_3) was measured by a competitive binding assay (Hummer *et al* 1984).

SCAN IMAGE

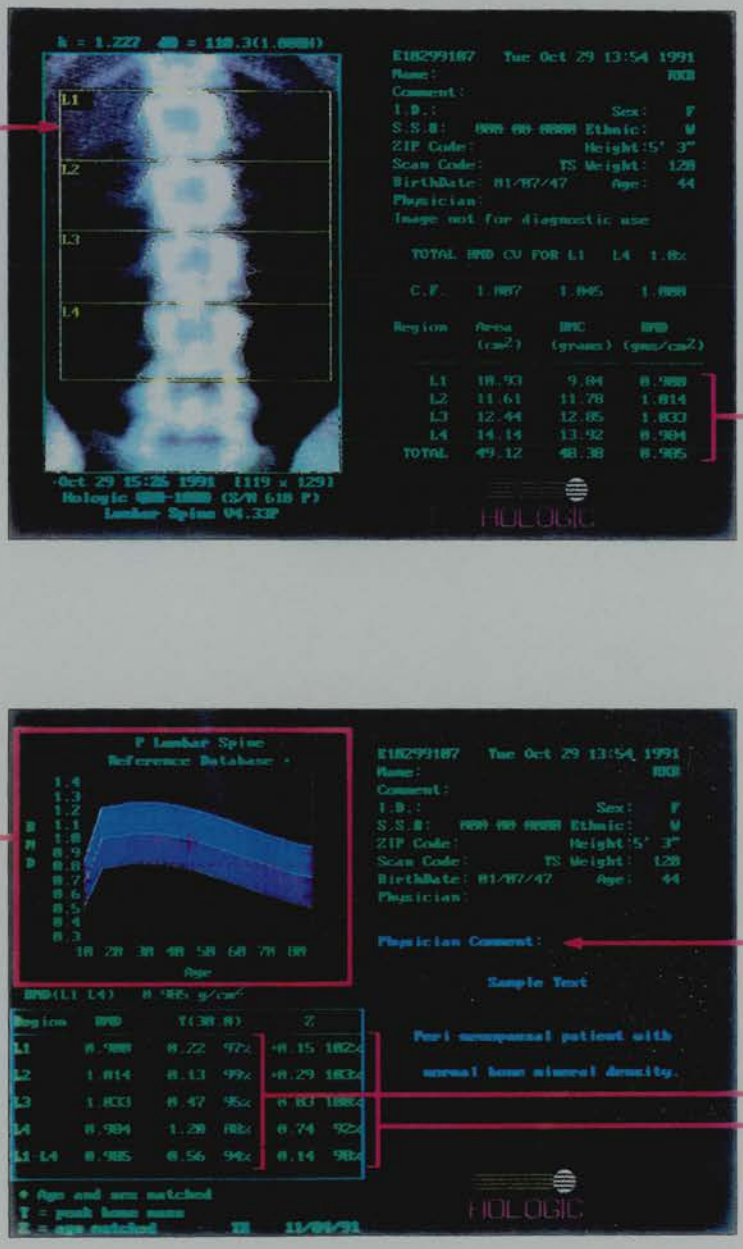
Global and individual vertebral regions-of-interest (ROI) are defined. The image can be visually checked for abnormalities that could compromise interpretation of the results, such as scoliosis, osteophytes, improper patient positioning, severe osteopenia and compression fractures. Individual vertebrae can be excluded from the scan if necessary.

REFERENCE DATABASE CURVE

The middle line separating the two shaded areas represents the mean BMD value as a function of age.

The light band above this line represents two standard deviations (SD) above the mean; the dark band below represents two SD below the mean.

The patient's BMD (+ symbol) is compared against young normals to show bone mineral loss since peak bone density, and against mean values for a peer group matched by age and sex.



BMD

The patient's bone mineral density (BMD) is expressed in g/cm². The total is the average of the regions included in the ROI. This measurement is plotted on the reference database curve (+ symbol) and can be tracked with serial scans to determine rate of bone loss or gain over time.

PHYSICIAN COMMENT

This area of the reference database screen is reserved for physician comments about the patient's scan values. The information can be stored permanently with the scan record on archive media.

T SCORE

The T score indicates the amount of bone loss, by quantifying the difference between the patient's BMD at his/her current age, and the peak bone mass for young normals. The young normals age is noted in parenthesis.

Z SCORE

The Z score normalizes the patient's BMD in a different way, by assessing the amount of bone loss compared to the expected loss for age matched peers.

Figure 3.2. An example of a spine DEXA scan. L1-L4 vertebrae are imaged. The information provided by the printout are highlighted.

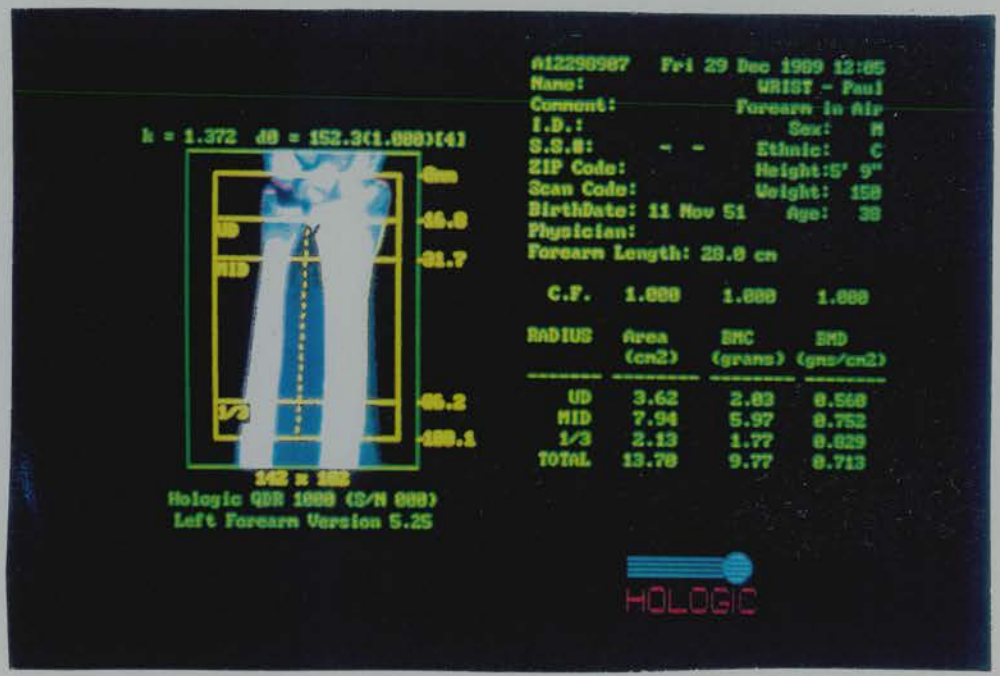
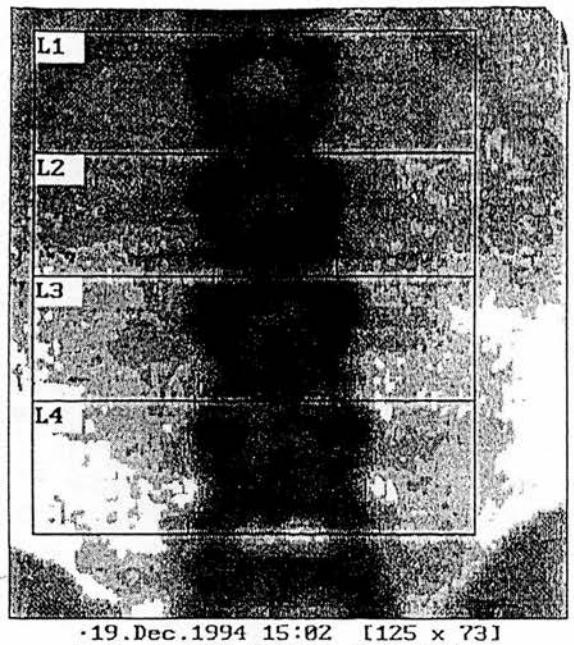


Figure 3.3. An example of a forearm DEXA scan. The three regions-of-interest as described in the text are illustrated.



·19.Dec.1994 15:02 [125 x 73]
Hologic QDR-1000/W (S/N 967 P)
Lumbar Spine V4.47Q

Comment:
I.D.: F03A018 Sex: M
S.S.#: - - Ethnic: W
ZIPCode: Height: 175.00 cm
Scan Code: CM Weight: 58.80 kg
BirthDate: 21.Feb.72 Age: 22
Physician: GHOSH

TOTAL BMD CV FOR L1 - L4 1.0%

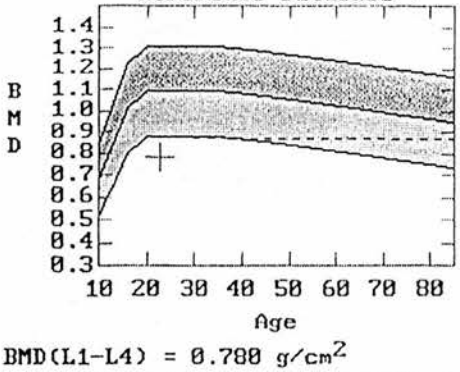
C.F. 1.017 1.108 1.000

Region	Area (cm^2)	BMC (grams)	BMD (gms/cm^2)
L1	13.73	9.57	0.697
L2	15.43	12.34	0.800
L3	16.43	13.67	0.832
L4	19.56	15.20	0.777
TOTAL	65.15	50.79	0.780

HOLOGIC

Medical Physics, WGH. Edinburgh.

Q Lumbar Spine
Reference Database •

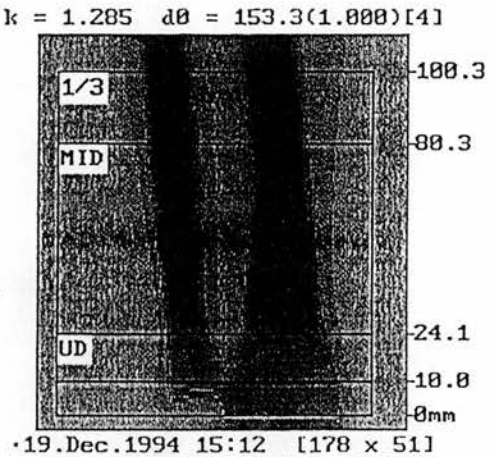


BMD(L1-L4) = 0.780 g/cm^2

Region	BMD	T(30.0)		Z	
L1	0.697	-2.83	69%	-2.83	69%
L2	0.800	-2.67	73%	-2.67	73%
L3	0.832	-2.46	75%	-2.46	75%
L4	0.777	-3.35	68%	-3.35	68%
L1-L4	0.780	-2.83	71%	-2.83	71%

• Age and sex matched
T = peak bone mass
Z = age matched TK 04 Nov 91

Figure 3.4. Spine BMD scan from a patient with long-standing Crohn's disease. He was osteopenic with a Z-score of -2.8.

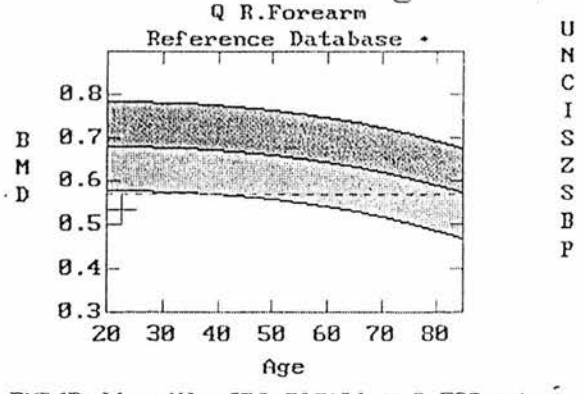


I.D.: F03A018 Sex: M
S.S.#: - - Ethnic: W
ZIPCode: Height: 175.00 cm
Scan Code: CM Weight: 58.80 kg
BirthDate: 21.Feb.72 Age: 22
Physician: GHOSH
Forearm Length: 27.5 cm

	C.F.	1.017	1.108	1.000
RADIUS + ULNA		Area (cm2)	BMC (grams)	BMD (gms/cm2)
1/3		6.27	4.35	0.693
MID		16.17	8.95	0.554
UD		6.40	1.97	0.308
TOTAL		28.84	15.27	0.529

HOLOGIC

Medical Physics, WGH Edinburgh.



BMD(Radius+Ulna[R] TOTAL) = 0.529 g/cm²

Region	BMD	T	Z
1/3	0.693	-2.31 84% (20.0)	-2.29 84%
MID	0.554	-2.57 80% (20.0)	-2.56 80%
UD	0.308	-3.60 60% (20.0)	-3.57 61%
TOTAL	0.529	-2.77 78% (20.0)	-2.76 78%

• Age and sex matched
T = peak bone mass
Z = age matched PS 25 Oct 91

Figure 3.5. Forearm BMD scan from the same patient as in figure 3.4. Osteopenia with a Z-score of -2.8 is illustrated.

3.3.3. iPTH

Plasma iPTH was measured by immunoradiometric assay (Nussbaum *et al* 1987) (Nichols Allegro immunoradiometric assay, Nichols Institute Diagnostics, Geneva, Switzerland). Plasma was separated within 15 minutes of blood collection and stored at -70°C.

3.4. Measurement of body composition

All had height measured without shoes (in cm) as described in detail by us (Ferguson *et al* 1994) and weight measured, wearing indoor light clothes on the same, accurately calibrated machine (in kg). In adult subjects, BMI was calculated by the formula:

$$BMI = \text{Weight (kg)}/\text{Height (m)}^2.$$

3.4.1. Dual Energy X-ray Absorptiometry (DEXA)

DEXA measurements to determine body composition were done with a Hologic QDR-1000W scanner (Hologic Inc., Waltham, MA, USA) operated in the total body mode. Analyses were done with the software version 5.51P. The effective radiation dose was 6μSv. Lean body mass was determined from the sum of bone and lean tissue components. The coefficient of variation of measurement of lean body mass was o.44%. The DEXA equipment was housed in a dedicated room with standard radiation protection measures.

3.4.2. Bioelectrical impedance

3.4.2.1. The machines

Bioelectrical impedance was measured using two machines:

1. A standard four terminal bioelectrical impedance plethysmograph (RJL systems Inc, model 101, Detroit, MI, USA), housed in and operated by staff of the Medical Physics Department (Figure 3.6). The machine gives a value for measured resistance, which is then used to calculate lean body mass on a personal computer, using a formula and software supplied by the manufacturer. This is hereafter referred to as the *in-house* machine.

2. Hand-held bioelectrical impedance machine (Bodystat-1500, Bodystat Ltd, Isle of Man, UK) body composition unit. This is a single frequency, lightweight, menu-driven bioelectrical impedance analyser that can store and recall data from 100 consecutive measurements (Figure 3.7). Results may be read out directly from the machine's display. The machine needs input of age, sex, height and weight, and directly displays lean body mass (as percentage of total body weight and in kg), fat mass (as percentage of total body weight and in kg), total body water (as percentage of total body weight and in litres), estimated basal metabolic rate and impedance in ohms. This is hereafter referred to as the *hand-held* machine.

3.4.2.2. The method

Impedance was measured between the right wrist and right ankle by a tetrapolar electrode method. The subjects were lightly clothed but without shoes or socks, and lay supine with arms separated from the body and legs not touching each other. Adhesive aluminium foil electrodes were positioned in the middle of the dorsal surface of the hands and feet proximal to the metacarpo- phalangeal and metatarsophalangeal joints (Figure 3.8 and figure 3.9); a second electrode was positioned more proximally between the distal prominence of the radius and the ulnar styloid and between the medial and lateral malleoli at the ankle. An excitation current of 800μA at 50kHZ was applied to the distal electrodes and the voltage drop was detected by the proximal electrodes.

3.4.2.3. Calculation of lean body mass from impedance value

The *in-house* machine had software supplied by the manufacturer for calculation of lean body mass. The hand-held machine was programmed to run on the manufacturer's prediction equation. The exact prediction equations used were not disclosed by the manufacturers. For patients with an eating disorder, a prediction equation based on the readings from the in-house impedance machine compared with DEXA data, had been previously derived in our institution by multiple stepwise regression (Hannan *et al* 1990):

Lean body mass (kg) = 0.344 W + 0.328 (H^2/R) + 0.576 SW - 9.63

where W is body weight (kg), H is height (cm), R is resistance (ohm) and SW is shoulder width (cm).

In the group of patients with an eating disorder, I used the value for impedance measured by the hand-held machine in this equation, and compared the result with that of the LBM derived automatically and displayed in the machine.

In IBD patients, the prediction equation derived by Jeejeebhoy's group (Royall *et al* 1994) was used in addition to the manufacturer's equation. This is as follows:

$$\text{Lean body mass} = \frac{0.25\ H^2/R + 0.29\ W + 3.63}{0.733}$$

3.4.2.4. Difference between *in-house* and *hand-held* machines

This is shown in table 3.1 along with the features of DEXA for comparison. The main advantage of the *hand-held* machine is its user-friendliness.

3.4.2.5. Precautions and caveats

Since lean body mass includes total body water, clinical conditions characterised by abnormal accumulation of fluid may render interpretation of the lean compartment difficult. Hence, ankle oedema or ascites were specifically looked for and patients manifesting these signs were excluded. Intake of food or emptying of the bladder has minimal effect on measurement of bioelectrical impedance and hence these variables were not controlled for.

Table 3.1. Characteristics of the different methods of body composition measurement

Feature	In-house BIA	Hand-held BIA	DEXA
Portable	yes	yes	no
Direct output of result	no	yes	no
Radiation	no	no	yes
Input height & weight	yes	yes	yes
Cost	£2200	£400	£80000
Weight	3.5Kg	420g	1200Kg
Trained operator	yes	no	yes

BIA= Bioelectrical impedance analysis

Bioelectrical Impedance Analysis (BIA)

50 kHz radiofrequency signal at 800 μA.

Controls: FFM from RJL prediction equation

Anorexics:

FFM (kg) = 0.344W + 0.328(H^2/R) + 0.576SW - 9.63

W = body weight (kg), H = height (cm),

R = resistance (Ω), SW = shoulder width (cm)

Figure 3.6. The *'in-house'* bioelectrical impedance plethysmograph (RJL systems inc. model 101) in the Medical Physics Department.

Figure 3.7. The '*hand-held*' bioelectrical impedance analysis machine (Bodystat-1500). Three simple buttons control all functions.

Figure 3.8. Method of bioelectrical impedance analysis. The subject lies supine and electrodes are connected to the hand and foot.

Figure 3.9. A close-up showing the position of the electrodes on the hand. The distal and proximal electrodes are colour-coded red and black respectively.

3.5. Measurement of urinary excretion of pyridinium crosslinks of collagen and plasma osteocalcin

Urine samples passed between 8-10am were collected on two consecutive days.from newly diagnosed patients with IBD described in chapter VI. These were stored at -20°C and then transported on dry ice to the Rowett Research Institute, Aberdeen. The assays were performed at the Biochemical Sciences Division. The method for the assays has been described in publications from this institute (Black *et al* 1988, Seibel *et al* 1989, Robins *et al* 1991). The pyridinium compounds measured were pyridinoline (Pyd) and deoxy-pyridinoline (Dpd) and the results were expressed as nmol/mmol creatinine, the latter being measured in unhydrolysed portions of the urine (Folin *et al* 1965).

For osteocalcin assays plasma was separated within 15 minutes of collection and stored at -70°C. All samples were collected between 8 am and 10 am. Frozen samples were transported to the Rowett Research Institute on dry ice. Plasma osteocalcin was measured by a radioimmunoassay.

3.6. Statistical methods

Statistical methods used for analysing results are mentioned in the results sections of the individual chapters that follow. Both BMD and body composition data had normal distributions and parametric statistics were used. The Minitab statistical software (release 8.2) was used for all calculations.

♦ ♦ ♦

Chapter IV

BONE MINERAL DENSITY IN CROHN'S DISEASE AND ULCERATIVE COLITIS AT DIAGNOSIS

4.1. Introduction

Low bone mineral content has been convincingly demonstrated in unselected groups of patients with IBD (Compston *et al* 1987, Stallmach *et al* 1988, Pigot *et al* 1992, Clements *et al* 1992) but the pathogenesis of the osteopenia is incompletely understood. No correlation between bone loss and serum parameters of bone metabolism has been demonstrated (Stallmach *et al* 1988). Though the deleterious effects of corticosteroids on trabecular bone mass are well documented (Smith 1990, Hahn *et al* 1974), longitudinal studies in patients with IBD have failed to show any significant correlation between the rate of bone loss and prednisolone therapy (Motley *et al* 1988). Conflicting evidence from cross-sectional studies however, indicated that cumulative corticosteroid use may be an important (Weldman *et al* 1993, Tromm *et al* 1993) or even the sole cause (Bernstein *et al* 1993) of osteopenia in patients with IBD. This is contradicted by other cross-sectional studies (Bjarnason *et al* 1993).

Various factors such as small intestinal resection (Hylander *et al* 1981), ileal involvement (Tromm *et al* 1993),vitamin D deficiency (Driscoll *et al* 1982), smoking (Weldmann *et al* 1993), body mass index (Motley *et al* 1988) and height (Issenman *et al* 1988) have been correlated with osteopenia in IBD reflecting the heterogeneous nature of the patient cohorts studied by various workers. It is still not known the extent to which bone mineral loss in IBD occurs as an integral manifestation of the disease as distinct from the secondary osteopenic influences of corticosteroid therapy, surgical intervention, inactivity, malabsorption of calcium and vitamin D or the cachexia of inflammation.

There has been no comparative study on bone mineralisation in patients with Crohn's disease (CD) and ulcerative colitis (UC). CD may be considered a systemic disease whereas UC is more limited to colonic mucosal inflammation; CD also has important immunological differences from UC (Shanahan 1993).

I have studied bone mineral density (BMD) in a series of newly diagnosed patients with CD and UC. Both spine and forearm BMDs were studied so that trabecular and cortical mineralisation could be assessed, as there are metabolic differences between these two types of bones (Compston 1990). Newly diagnosed

patients were studied in order to avoid most of the secondary osteopenic influences mentioned previously. The measurement of BMD was repeated about a year after diagnosis to assess progressive bone loss and the effects of therapy.

4.2. Materials and Methods

4.2.1. Subjects

A total of 30 consecutive patients (17 men, 13 women) aged 14-83 years (median=26 years) with newly diagnosed IBD were studied between March 1991 and August 1993. The diagnosis and disease extent in all patients were confirmed by histology, endoscopy and radiology. In 15 patients (9 men, 6 women) aged 14-83 years (median=24 years) CD was diagnosed. In another 15 patients (8 men, 7 women) aged 14-63 years (median=28 years) UC was diagnosed. The distribution of disease is given in table 4.1.

Table 4.1. Macroscopic distribution of disease in newly diagnosed IBD patients

Diagnosis and anatomy	Male	Female
CD	9	6
Colonic	6	3
Ileal	1	2
Ileocolonic	1	1
Jejunal	1	0
UC	8	7
Pancolitis	4	3
Distal colitis	1	0
Proctitis	3	4

The logistics of setting up BMD studies normally meant a delay of 2-3 weeks. For ethical reasons, we did not attempt to delay appropriate medical treatment during this interval. As a result, BMD measurements were made in 14 of the 30 patients shortly after the start of oral corticosteroids; in 13 patients the duration ranged from 4-14 days and in one case 19 days.

4.2.2. Clinical assessments

The patients were clinically assessed by me and each patient completed a diary card for a week prior to bone density measurements for calculation of Crohn's

disease activity index (CDAI) (Best *et al* 1976). The haematocrit for CDAI was obtained from the full blood count. For patients with UC, both CDAI and Powell-Tuck index (PTI) (Powell-Tuck *et al* 1978) were calculated. Smoking status, bone fracture history and experience of contraceptive pill and hormone replacement therapy were recorded. CDAI with or without PTI were again calculated just prior to follow-up measurement of bone density. Height and weight were recorded and the body mass index (BMI) was calculated as weight (kg) divided by height (metre)^2. The estimated duration of disease in weeks before diagnosis was also recorded. In one 28-year-old male UC patient, forearm BMD was not available.

Bone density measurements were done after diagnosis and at follow-up 1 year later.

4.2.3. Methods

Assessment of physical activity and biochemical measurements are described in chapter III. BMDs at lumbar 1-4 vertebrae and right forearm were measured by DEXA as detailed in chapter III.

4.2.4. Statistical Analysis

Comparison between CD and UC was made using the unpaired t-test. Comparison of initial measurements with follow-up measurements was made by the paired t-test. Correlation coefficients were calculated with the Pearson's correlation test.

4.2.5. Ethical considerations

The study was approved by the Medicine Subcommittee of the Lothian Area Ethics of Research Committee. Each patient gave informed verbal consent.

4.3. Results

The age and sex distribution, duration of symptoms prior to diagnosis, physical activity grade and duration of steroid use prior to diagnosis for CD and UC are summarised in table 4.2. The mean spine Z-score for 17 male patients was -0.56 (SD 1.07) and that for 13 female patients was -0.48 (SD 1.25; p=NS). The mean of the 12 forearm Z-scores available from male patients was not different from the mean of 9 forearm Z-scores available from female patients (-0.41+1.49 vs -0.66+0.99; p=NS).

Table 4.2. Clinical features of CD and UC

Features	CD	UC
Age (year)	24 (14-83)	28 (14-63)
Sex (M:F)	9:6	8:7
Estimated duration of disease before diagnosis (weeks)	18.6 (18.6)	12.3 (15.5)
Duration of steroid use before BMD measurement (weeks)	1.2 (1.1)	0.5 (0.8)
Grade of physical activity		
Premorbid	3.7 (0.6)	3.9 (0.3)
Active disease	2.8 (0.6)	3.1 (0.9)
BMI	21.38 (3.17)	22.36 (4.33)
Active disease (CDAI > 150 or Powell-Tuck index >4)	11	10

Note: All values are expressed as mean (SD) except age, which is given as median (range).

The mean BMIs in patients with CD and UC are also given in table 4.2. The spine and forearm Z-scores obtained from bone mineral content per unit area expressed as standard deviations from the age and sex matched means were not significantly correlated with BMI (r=0.20 and 0.11 respectively). However, spine and forearm total bone mineral content significantly correlated with BMI (r=0.76 and 0.75; $p<0.05$) which implied that increasing BMI was associated with larger bones and hence a larger area of interest.

Four patients (2 UC, both females; 2 CD both males) were less than 16 years old. The UC patients were on the 90th and greater than 97th centile in height.The CD patients were on the 90th and 3rd centile in height. The 14-year-old boy with CD with height on the 3rd centile had a bone age of 12. The bone age of the remaining three adolescents were appropriate for age. A further 4 patients (3 UC, 1 CD) were aged 18-19 years and none of them were short-statured (2 male UC=182 and 178cm; 1 female UC=157cm; 1 female CD=155cm) - their bone ages were that of mature adults. The mean height of 7 men aged 20 years and over with CD (173cm SD 4cm) was not different from the mean height of 6 men aged 20 years and over with UC (173cm SD 5cm). The mean height of 5 women aged 20 years and over with CD (158cm SD 6cm) was not significantly different from the mean height of 4 women aged 20 years and over with UC (166cm SD 4cm).

The mean CDAI in patients with CD was 196 (SD 70). CDAI was calculated for patients with UC and this was not different from that for patients with CD (mean 164,

SD 105; p=NS). Eleven out of the fifteen patients with CD had a CDAI over 150 indicating active disease. Ten out of the 15 patients with UC had a PTI > 4 indicating active disease (table 4.2). There was no correlation between the CDAI and spine or forearm Z-score (r= -0.17 and r= -0.20 respectively) in patients with CD. Likewise, there was no correlation between spine or forearm Z-score and the PTI (r= -0.26 and -0.22 respectively) in patients with UC. In the entire cohort of IBD patients, indices of systemic inflammatory activity such as CRP (r= -0.3, p=NS), platelet count (r=0.1, p=NS) and white cell count (r= -0.2, p=NS) were not significantly correlated with spine BMD Z-scores.

Nine patients with CD had colonic involvement only and 6 patients with CD had small bowel involvement. The spine and forearm Z-scores in these two groups of patients are depicted in table 4.3. The Z-scores for the two CD groups with different anatomical regions involved did not differ significantly.

Table 4.3. Z-scores in CD according to region of involvement

Site	Colonic {mean (SD)}	Small bowel {mean (SD)}
Spine Z score	-1.09 (1.12) (n=9)	-1.02 (0.41) (n=6)
Forearm Z score	-0.86 (0.67) (n=8)	-1.35 (1.17) (n=4)

Fourteen out of the 30 patients were smokers and the remainder were non or ex-smokers. The mean spine Z-score in the 14 smokers was -0.26 (SD 1.15) and in the 16 non/ex-smokers was -0.74 (SD 1.11). The difference was not significant. The forearm Z-scores were available for 10 smokers and 11 non/ex smokers; the mean Z-scores were not different (-0.58+0.65 vs -0.47+1.51; p=NS). Among patients with CD, eight were smokers and their spine and forearm Z-scores were not different from those of the non-smokers.

Two patients with CD were on hormone replacement therapy for more than 5 years and their spine Z-scores were -2.9 and -1.0. In neither case was bone disease the reason for prescribing hormone replacement therapy. Secondary amenorrhoea was present in 1 CD patient (spine Z-score=-1.5) and 1 UC patient (spine Z-score= -1.1). Three patients with CD were on oral contraceptives (spine Z-scores= -0.6, 0.6, -1.2) and 4 patients with UC were on oral contraceptives (spine Z-scores= -0.4, -0.5, 1.3,-1.2).

Table 4.4 depicts the biochemical parameters in CD and UC. None of the parameters was significantly different in the two groups of patients. Plasma Ca^{++} levels were low in two patients with severe UC, both of whom had low plasma albumin

levels; corrected Ca^{++} levels were normal. Though urinary hydroxyproline excretions were above the upper limit of normal (168 umol/24hr/sq.cm of body surface) in 8 out of the 30 patients, urinary hydroxyproline/creatinine ratios were within normal limits in all patients.

Table 4.4. Biochemical markers of calcium homeostasis

Bone biochemistry (normal range)	CD {mean (SD)}	UC {mean (SD)}
Total calcium (mmol/L)* (2.25 - 2.50)	2.29 (0.04)	2.30 (0.04)
Urinary Ca^{++}/creatinine	0.26 (0.20)	0.29 (0.39)
Plasma PO_4 (mmol/L) (0.87 - 1.45)	1.11 (0.16)	1.22 (0.17)
Urinary PO_4/creatinine	0.50 (0.29)	0.80 (1.12)
Urinary OH-proline/creatinine	16.07 (4.46)	15.93 (3.88)
25-OH vitamin D (nmol/L)	36 (11)	41 (13)
Summer (15-100)	45 (12)	47 (14)
Winter (15-50)	28 (10)	37 (11)
Alkaline phosphatase (U/L)	45 (12)	47 (14)
Adult (30-140)		
Children (250-800)		
Immunoreactive parathyroid hormone (ng/L) (10-55)	39 (15)	41 (17)

* Total calcium corrected for albumin where appropriate

4.3.1. Bone density of lumbar spine

Fig. 4.1 shows the lumbar BMD values related to age in patients with CD and UC separately for males and females. The mean spine BMD (0.88+0.17) in patients with CD was significantly less than that in patients with UC (1.03+0.14; $p<0.02$). The rest of the comparisons between CD and UC are based upon Z-scores as these are indexed against age and sex matched controls.

Fig.4.2 shows the Z-scores of lumbar spine for patients with CD and UC. The mean spine Z-score for patients with CD was -1.06 (SD 0.86) while the mean Z-score of patients with UC was -0.03 (SD 1.16). This difference is significant ($p<0.02$). The mean Z-score of the 7 patients with proctitis was 0.16 (SD 1.2) and this was not different from the mean Z-score of the 8 patients with more extensive UC (-0.2 + 1.17; p=NS).

The 14-year-old boy with height on the 3rd centile had his spine Z-score recalculated using his bone age. This altered his spine Z-score from -1.5 to -0.5 but did not alter the significance level of the difference between CD and UC ($p<0.02$).

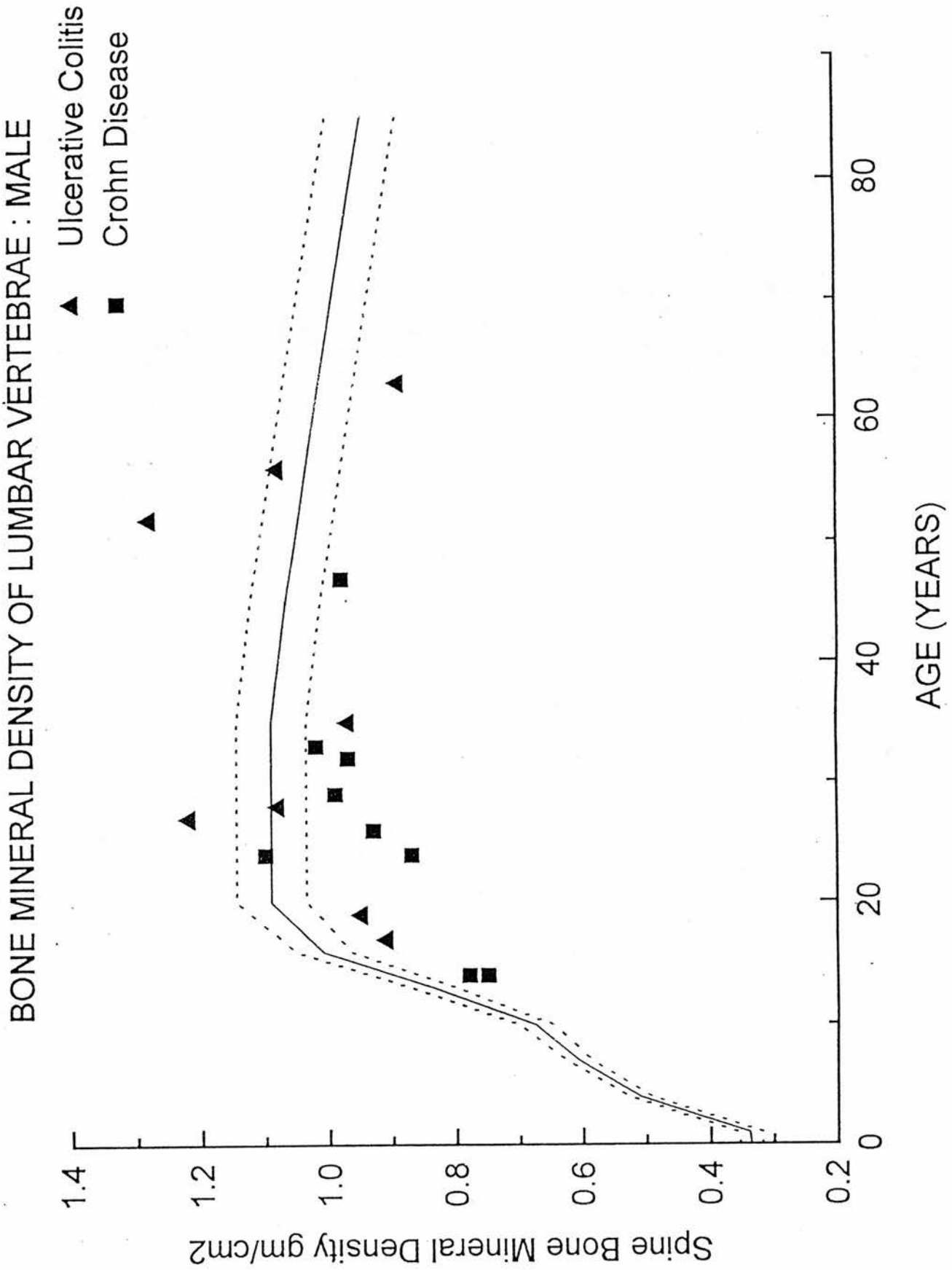


Figure 4.1a. BMD of lumbar vertebrae L1-L4 in male patients with IBD. Squares represent values for patients with CD and triangles represent values for patients with UC. The dotted lines indicate 1 standard deviation from the mean.

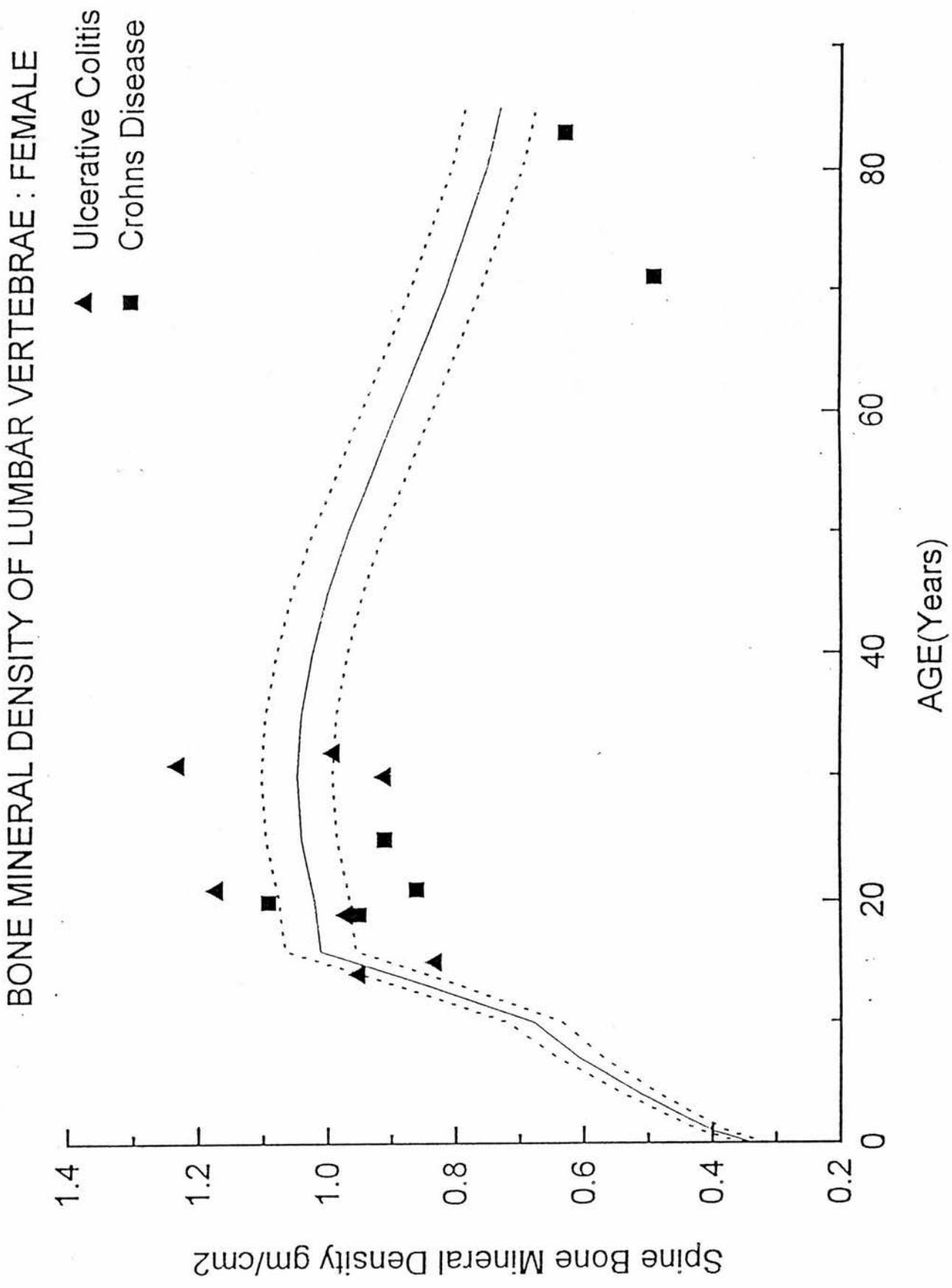


Figure 4.1b. BMD of lumbar vertebrae L1-L4 in female patients with IBD. Squares represent values for patients with CD and triangles represent values for patients with UC. The dotted lines indicate 1 standard deviation from the mean.

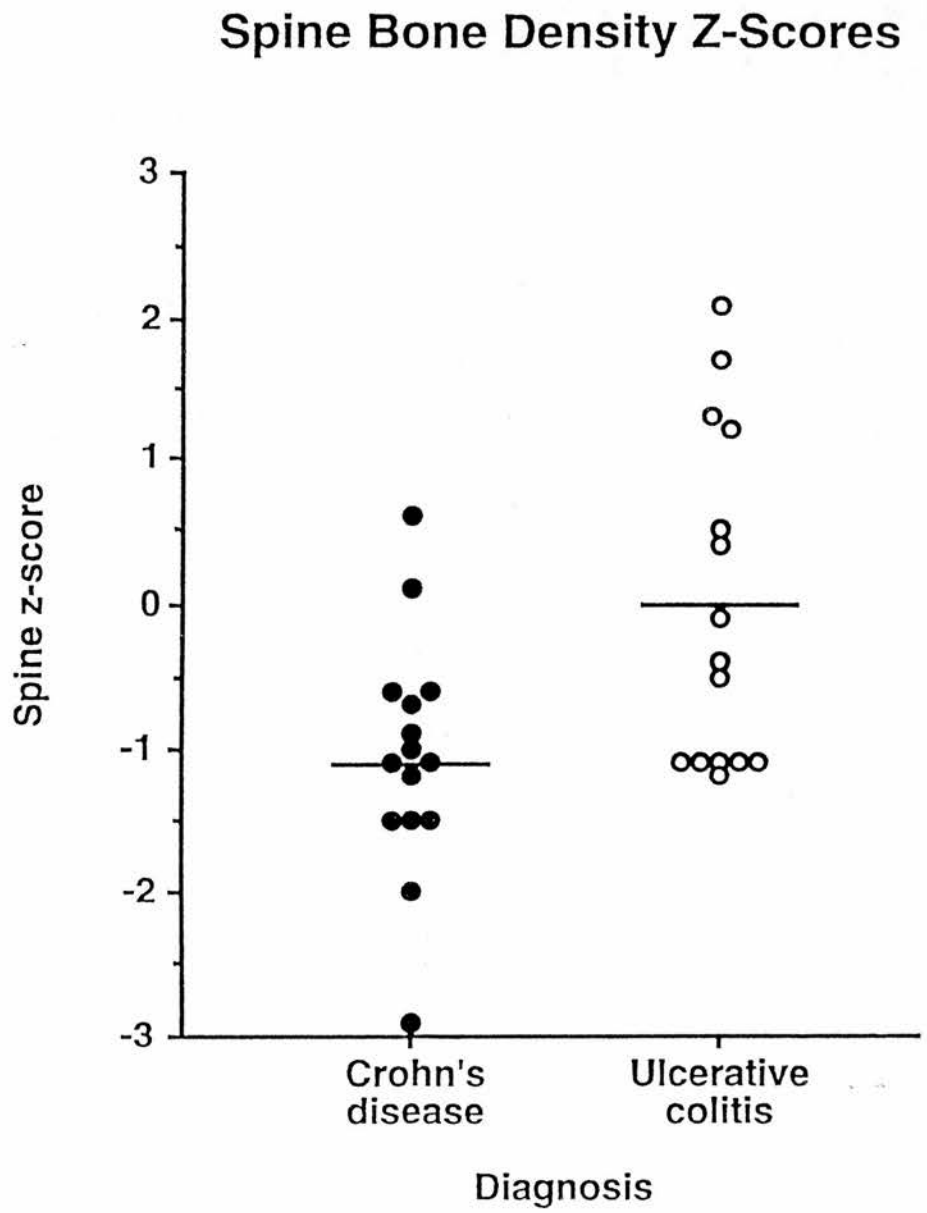


Figure 4.2a. Lumbar spine BMD Z-scores in patients with CD and UC at diagnosis. The horizontal bars represent mean values.

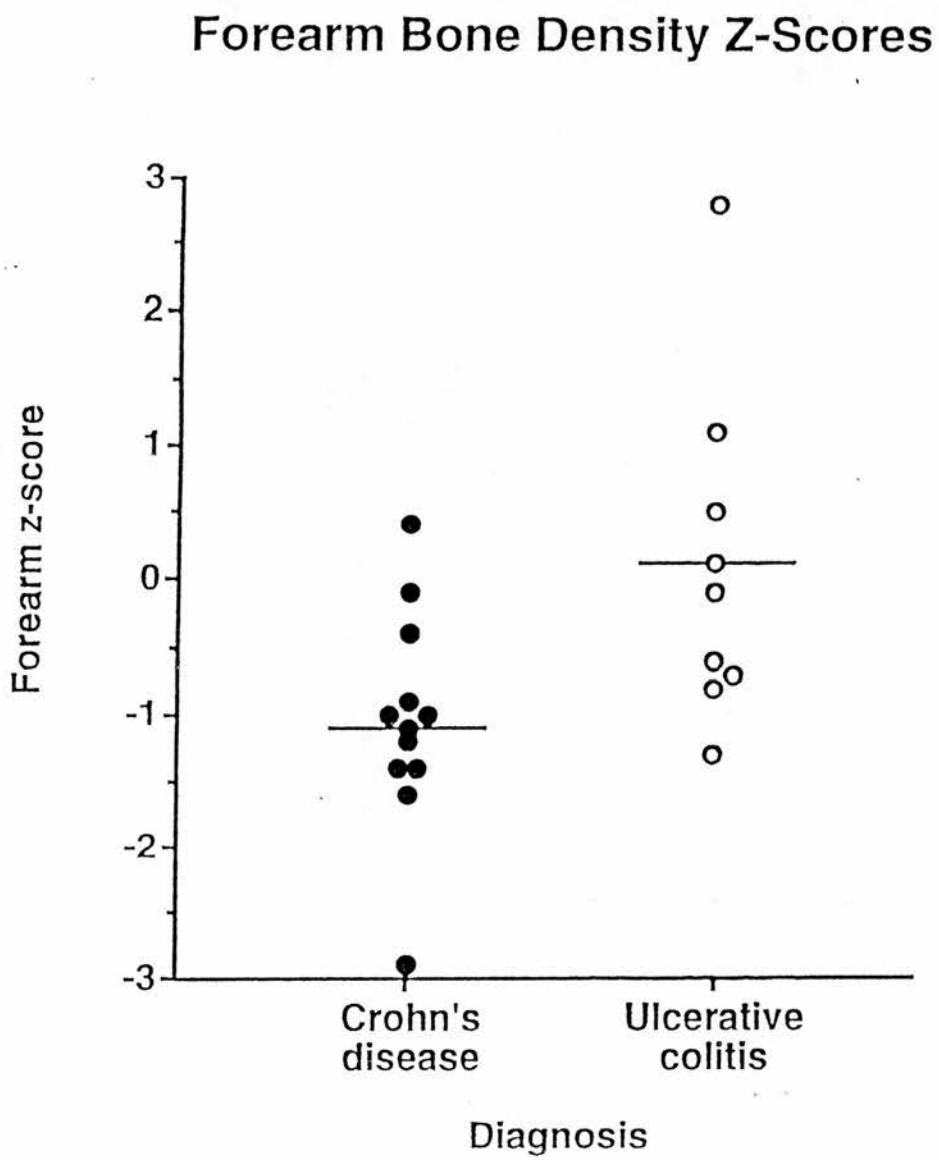


Figure 4.2b. Forearm BMD Z-scores (mean of the three sites of measurement) in patients with CD and UC at diagnosis. The horizontal bars represent mean values.

4.3.2. Bone density of forearm

Figure 4.3 shows the forearm BMDs for patients with CD and UC separately for males and females. Forearm Z-scores were available for 12 patients with CD and for 9 patients with UC who were aged 20 years or more (figure 4.2). The mean forearm Z-score for patients with CD disease was -1.04 (SD 0.86) while the mean forearm Z-score for patients with UC was 0.11 (SD 1.24). This difference is significant ($p<0.05$).

The three different measurement sites of the forearm were separately analysed as shown in table 4.5. The Z-scores for all three sites were significantly lower for patients with CD compared to patients with UC.

Table 4.5. Z-scores for the three forearm BMD measurement sites in CD and UC

Diagnosis	One-third distal {mean (SD)}	Middistal {mean (SD)}	Ultradistal {mean (SD)}
CD	-1.67 (0.77)*	-1.43 (0.82)**	-0.60 ().98)*
UC	-0.70 (1.00)	-0.32 (1.19)	0.87 (1.53)

* $p < 0.02$; ** $p < 0.01$

4.3.3. Relation between forearm and spine bone density (Fig.4.4)

Forearm Z-scores were available for 21 out of the 30 patients studied. The forearm Z-scores of these patients were significantly correlated with spine Z-scores ($r=0.52$; $p=0.02$).

4.3.4. Follow-up

Follow-up data could be obtained in 11 out of the 15 CD patients.
All of them had received systemic steroids. In addition four patients had received rectal steroids. The mean cumulative corticosteroid dose between the initial and follow-up bone mineral density measurement was 2.82g (SD 3.75g). One patient was receiving azathioprine. Two patients had resectional surgery within this period (colectomy=1; ileal resection=1). The mean CDAI (86+58) had come down significantly ($p<0.02$). Only one patient had a CDAI above 150 at the time of follow-up. The mean physical activity grade at follow-up was 3.1 (SD 0.8) and this was not significantly different from that at diagnosis. The mean BMI at follow-up was 21.45 (SD 3.85) and this too was not significantly different from that at diagnosis.

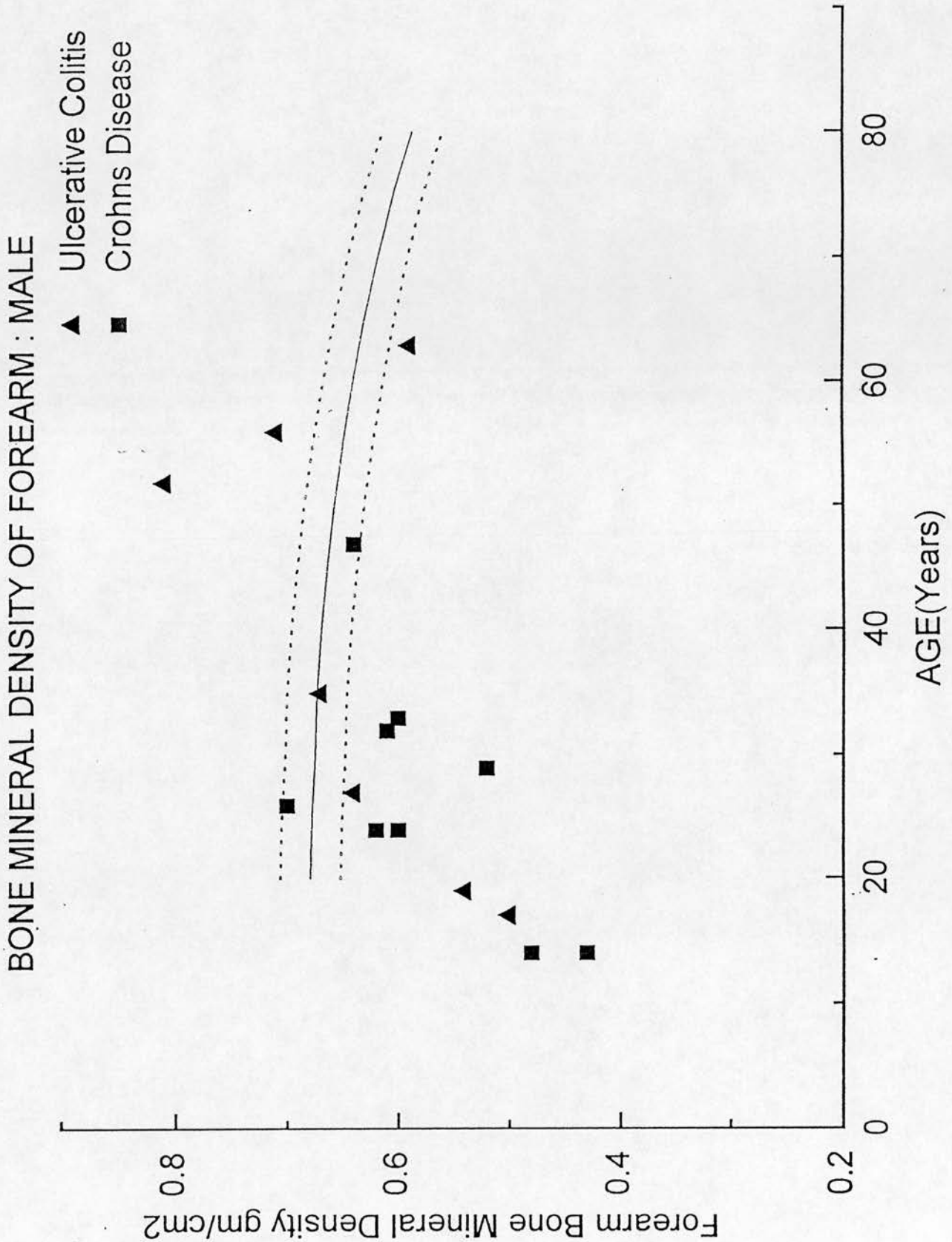


Figure 4.3a. BMD of forearm in male patients with IBD. Squares represent values for patients with CD and triangles represent values for patients with UC. The dotted lines indicate one standard deviation from the mean.

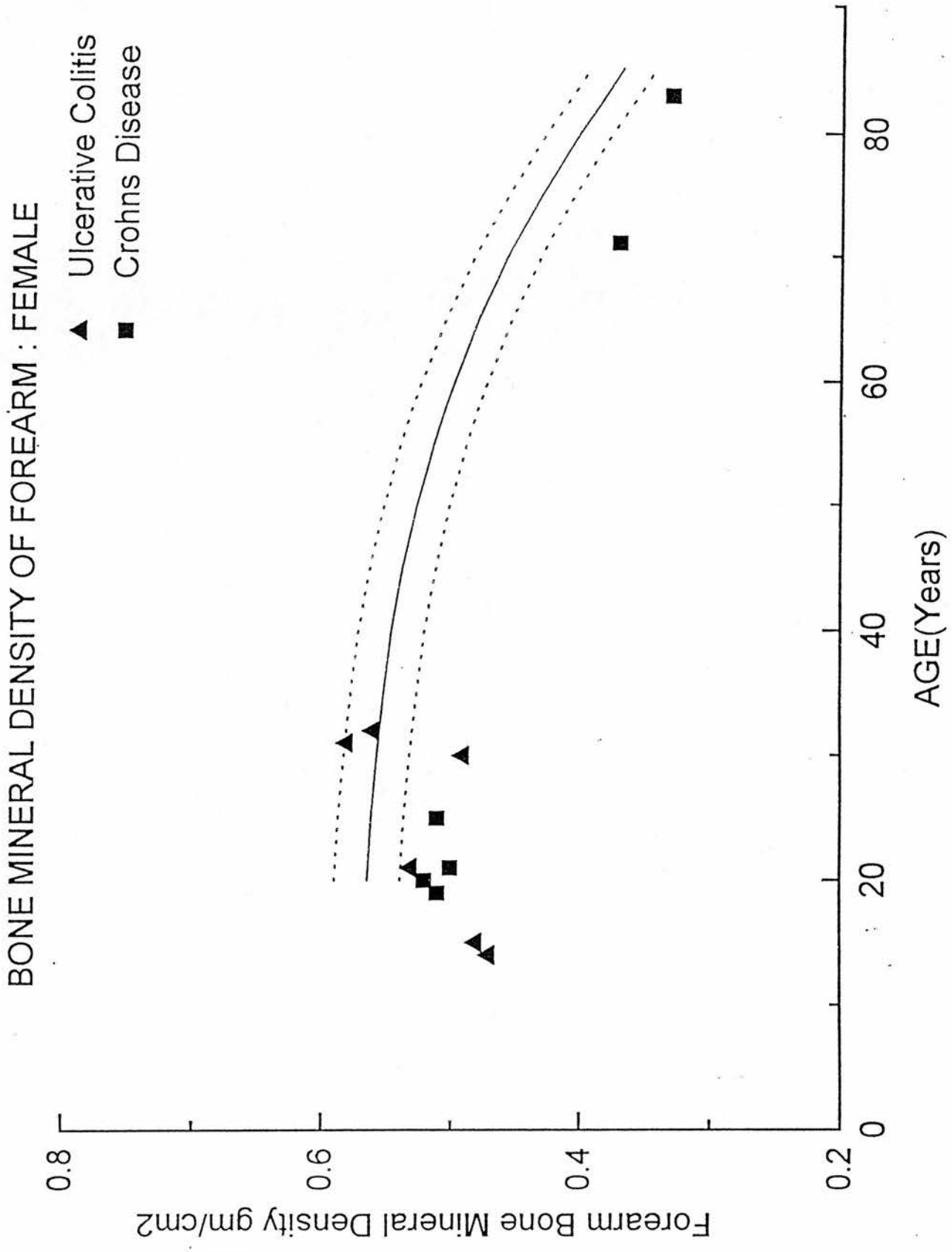


Figure 4.3b. BMD of forearm in female patients with IBD. Squares represent values for patients with CD and triangles represent values for patients with UC. The dotted lines indicate one standard deviation from the mean.

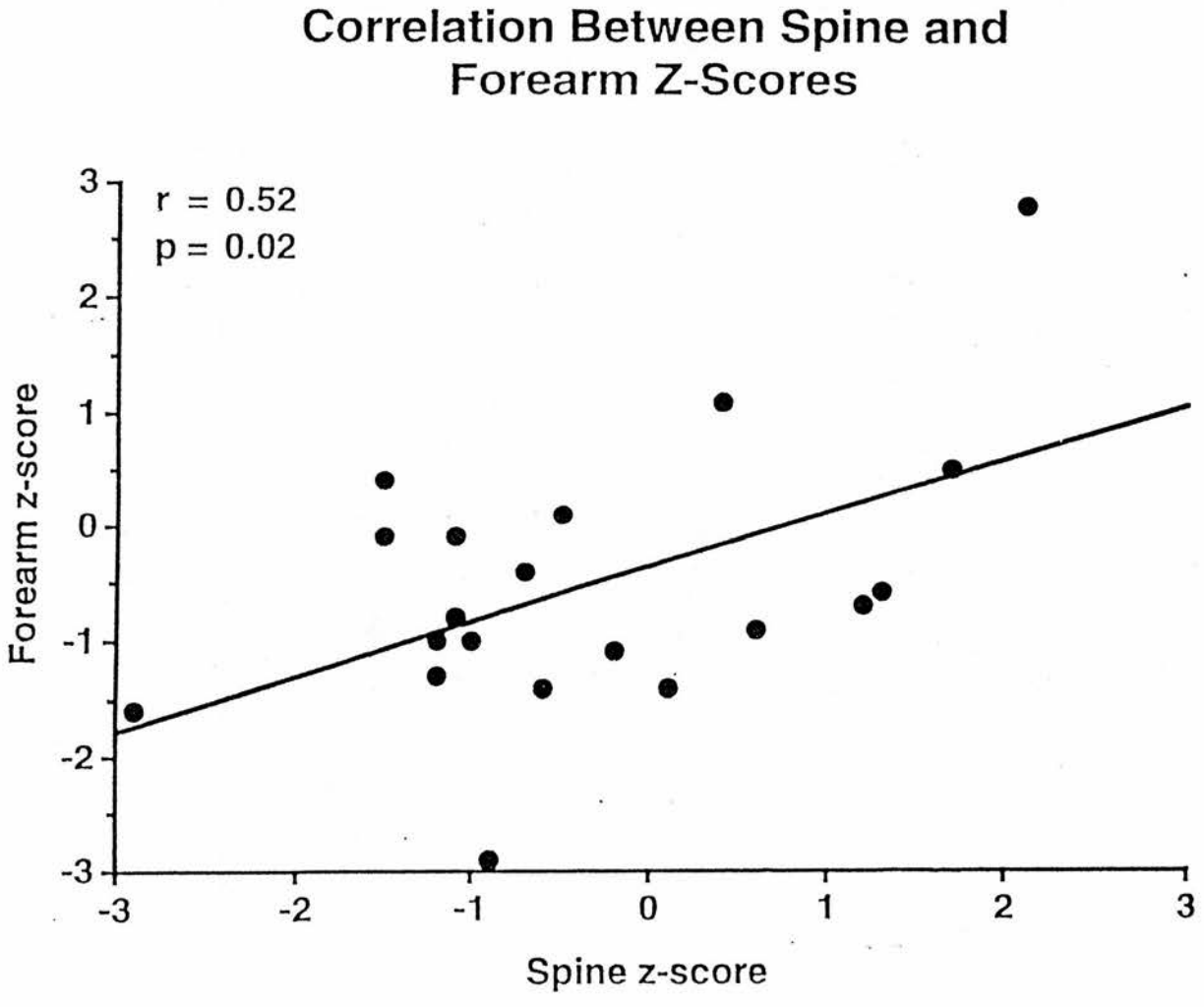


Figure 4.4. Correlation between forearm and spine BMD Z-scores in the 30 patients with IBD.

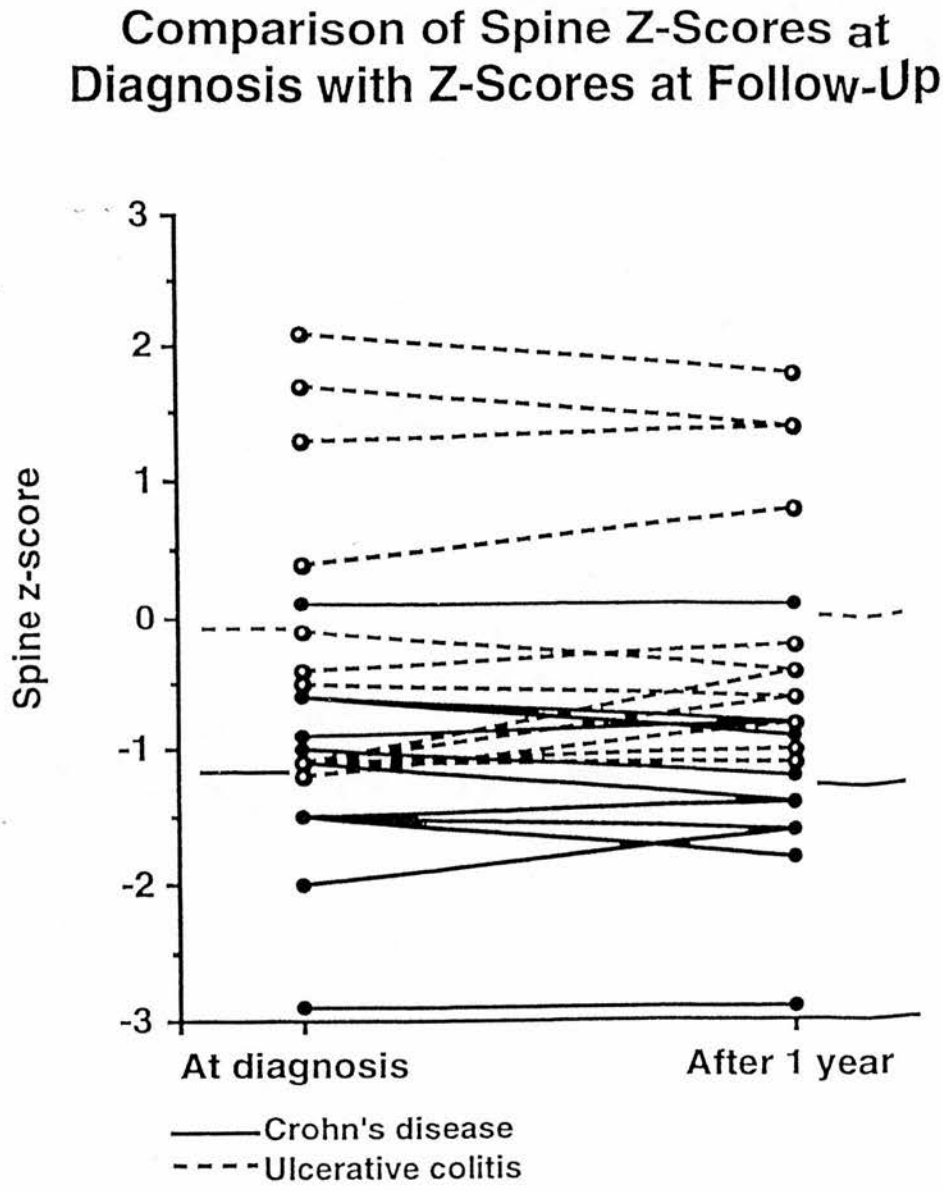


Figure 4.5a. Comparison of lumbar spine BMD Z-score values at diagnosis with Z-score values at follow-up after one year. Continuous lines (------) represent CD and broken lines (- - -) represent UC. The horizontal bars represent mean values.

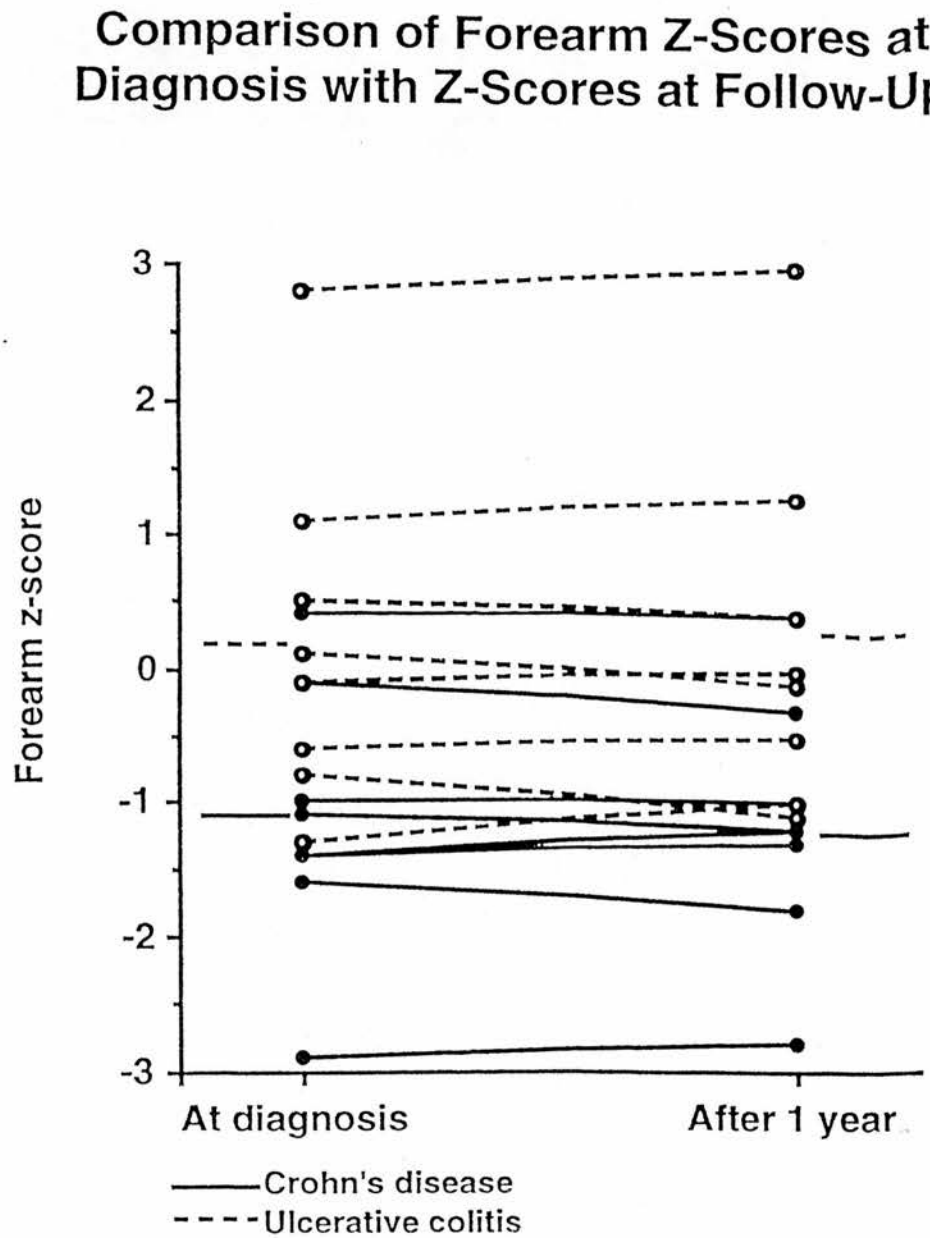


Figure 4.5b. Comparison of forearm BMD Z-score values at diagnosis with Z-score values at follow-up after one year. Continuous lines (------) represent CD and broken lines (- - -) represent UC. The horizontal bars represent mean values.

In these 11 patients with CD a repeat measurement of bone density was performed after 1 year (fig. 4.5). The mean spine Z-score after 1 year was -1.37 (SD 0.82) and this was not significantly different from the mean spine Z-score at diagnosis.

Forearm Z-scores were available in 8 of these patients and the mean score was -1.17 (SD 0.95); this too was not significantly different from initial measurements. In the single patient with CDAI over 150, spine and forearm Z-scores at follow-up were -1.6 and 0.4 respectively compared with -1.5 and 0.4 respectively at diagnosis.

Follow-up data were available for 12 out of the 15 UC patients. Nine out the 12 patients with UC had received systemic steroids and rectal steroids while the remaining 3 patients had received rectal steroids only. The mean cumulative systemic corticosteroid use over this period was 2.25g (SD 2.80g). The mean physical activity grade was 3.4 (SD 0.7) and the mean BMI 22.50 (SD 4.33) and none of these was significantly different from the relevant value at diagnosis. Two patients underwent panproctocolectomy with ileoanal pouch during this period. The mean PTI had come down to 3.4 (SD 2).

Twelve patients with UC underwent repeat bone density measurements Figure 4.5). The mean spine Z-score a year after the initial measurement was 0.04 (SD 1.06) and the mean forearm Z-score (9 patients) was 0.11 (SD 1.07). None of these were significantly different from previous measurements.

Both spine and forearm Z-scores continued to be significantly lower in CD compared with UC ($p<0.02$).

4.4. Conclusion

We have found that BMD of lumbar vertebrae and forearm is reduced at diagnosis in patients with CD compared with UC. This is unassociated with abnormalities of calcium homeostasis, BMI, smoking or prednisolone use. Treatment with corticosteroids does not necessarily result in further bone mineral loss. However, owing to the small number of patients studied, the likelihood of identifying any single factor that might have influenced the BMD of patients with CD versus those with UC is low. The potential influence of smoking or menstrual status could not be studied by discriminant analysis. This is discussed further in chapter X.

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Chapter V

BONE MINERAL DENSITY IN ADULT PATIENTS WITH LONGSTANDING INFLAMMATORY BOWEL DISEASE.

5.1. Purpose of this study

None of the previous studies on bone mineral density (BMD) in IBD patients had documented any difference between Crohn's disease (CD) and ulcerative colitis (UC). However, none of these studies had recruited only newly diagnosed patients as was the case for the work described in the previous chapter. A survey of the results from previous studies clearly showed that their cohorts of IBD patients had long-standing disease, as shown in the table 5.1 below. It is likely that in such cohorts, many confounding variables will have been present , particularly, prolonged steroid therapy and surgical intervention along with complex nutritional deficiencies, so that any primary effect of the disease would be masked.

5.1.1.Results of previous studies (table 5.1)

Table 5.1. Duration of disease in IBD patients included in previous studies

Authors(year)	No of CD & UC patients	Average duration of disease
Compston et al 1987	45 CD, 17 UC	10yrs
Clements et al 1992	33 CD, 17 UC	7.9yrs
Pigot et al 1992	27 CD, 34 UC	5yrs (CD), 6yrs (UC)
Clements et al 1993	22 CD, 25 UC	12yrs
Abitbol et al 1995	34 CD, 50 UC	6.3yrs
Bernstein et al 1995	50 CD, 24 UC	15yrs (CD), 9yrs (UC)

5.1.2. Selection bias:

If unselected patients are recruited from outpatient clinics of a tertiary referral hospital, it is probable that the cohort will be biased towards patients with long-standing, complicated disease because such patients attend for consultation most frequently and will contribute inappropriately to a cross-sectional cohort. The study design and the cohort characteristics of the studies represented in the table above would suggest that such a selection bias had occurred.

In the study described in this chapter, I present the results of BMD measurements in a cohort of patients with long-standing CD and UC. I wanted to test the hypothesis that in patients with long-standing IBD, there is no difference in BMD between CD and UC, unlike that at diagnosis.

5.2. Patients

5.2.1. Entry criteria

The following criteria were set for patient recruitment:

1. Disease duration from diagnosis > 5 years
2. Inactive disease (CDAI<150 or Powell-Tuck index<4)
3. Duration of steroid therapy≥6 months
4. Age 20-65 years

5.2.2. Exclusion criteria

1. Cholestatic liver disease such as primary sclerosing cholangitis, thyroid disorders, renal failure, hypogonadism.
2. Drugs such as hormone replacement therapy, thyroxine.

5.2.3. Patients

Fourteen patients with CD and 13 patients with UC were recruited in the study. Their characteristics are described below:

Table 5.2. Clinical characteristics of chronic IBD patients

Features	CD	UC
Age (years)	34 (22-65)	33 (21-64)
Sex (M/F)	8/6	7/6
Duration of disease (years)	8 (5-18)	7 (5-14)
Cumulative steroid dose. Median (range) gm	9.5 (3.6 - 20.2)	8.6 (3.2 - 18.9)
Duration of steroid use (months)	21 (8-45)	19 (7-42)
Grade of physical activity	3.4 (2-4)	3.7 (2-5)
BMI	21.2 (18.1-24.6)	22.8 (20.2-31.1)
No. of resectional operations. Median (range)	2 (1-6)	<1 (0-2)

Out of the 14 CD patients, 7 had colonic disease, 6 had small bowel disease and 1 patient had involvement of both the small bowel and colon. Of the 13 patients with UC, 10 had pan or extensive colitis and 3 had left sided colitis.

5.3. Methods

BMDs at lumbar spine and forearm were assessed by DEXA as described in chapter III. Biochemical tests of calcium homeostasis were less extensive than in the newly diagnosed patients. The tests done comprised plasma calcium, phosphate (non-fasting), alkaline phosphatase, albumin and 25-OH-vitamin D. A number of previous studies (Compston *et al* 1987, Pigot *et al* 1992, Abitbol *et al* 1995, Hessov *et al* 1984) had extensively investigated calcium homeostasis parameters in patients with long-standing inflammatory bowel disease and found no significant abnormalities. However, low levels of vitamin D had been found commonly in patients in some other studies (Driscoll *et al* 1982, Vogelsang *et al* 1989) and hence this was monitored. BMD was also measured in 10 patients with CD and 4 patients with UC who did not fulfill criteria for the study as they did not have a disease duration after diagnosis of > 5 years. The BMD of these patients, as well as the newly diagnosed patients described in chapter IV were utilised to analyse the correlation between disease duration and BMD. These data are presented at the end of the results section of this chapter.

Comparisons between UC and CD were made by the Student's t-test. The study was approved by the Medicine Subcommittee of the Lothian Area Ethics of Research Committee.

5.4. Results

The age and sex distributions, duration of the disease from diagnosis and physical activity were similar in UC and in CD. The spine BMD in the 15 male patients (mean -1.2, SD 0.8) was not significantly different from that in the 12 female patients (mean -0.9, SD 0.9, p=NS). Similarly, the forearm BMD in the 15 male patients (mean -1.1, SD 1.3) was not significantly different from that in the 12 female patients (mean -0.8, SD 0.7, p=NS). None of the female patients was on hormone replacement therapy. Two patients, one patient with CD and one with UC were on an oral contraceptive pill. Three patients with CD and two with UC were

postmenopausal. All patients had inactive disease as defined on the basis of CDAI or Powell-Tuck index since this was a criterion for their inclusion.

5.4.1. Spine Z-scores (Figure 5.1)

The mean Z-score for spine BMD in CD was -1.1 (SD 1.0) and in UC was -1.0 (SD 0.6) p=NS. Compared to the newly diagnosed IBD patients described in chapter IV, the mean spine Z-score in patients with long-standing CD was not significantly different, whereas in UC the difference was significant ($p<0.02$).

5.4.2. Forearm Z-scores (Figure 5.2)

The mean Z-score for forearm BMD in CD was -1.0 (SD 1.3) and in UC was -1.0 (SD 0.6), p=NS. The forearm Z-scores in newly diagnosed UC patients described in chapter IV was significantly higher compared to this cohort of long-standing UC patients ($p<0.05$), whereas there was no difference between newly diagnosed and long-standing CD. Two patients, both with CD, had spine BMD Z-score lower than -2.5.

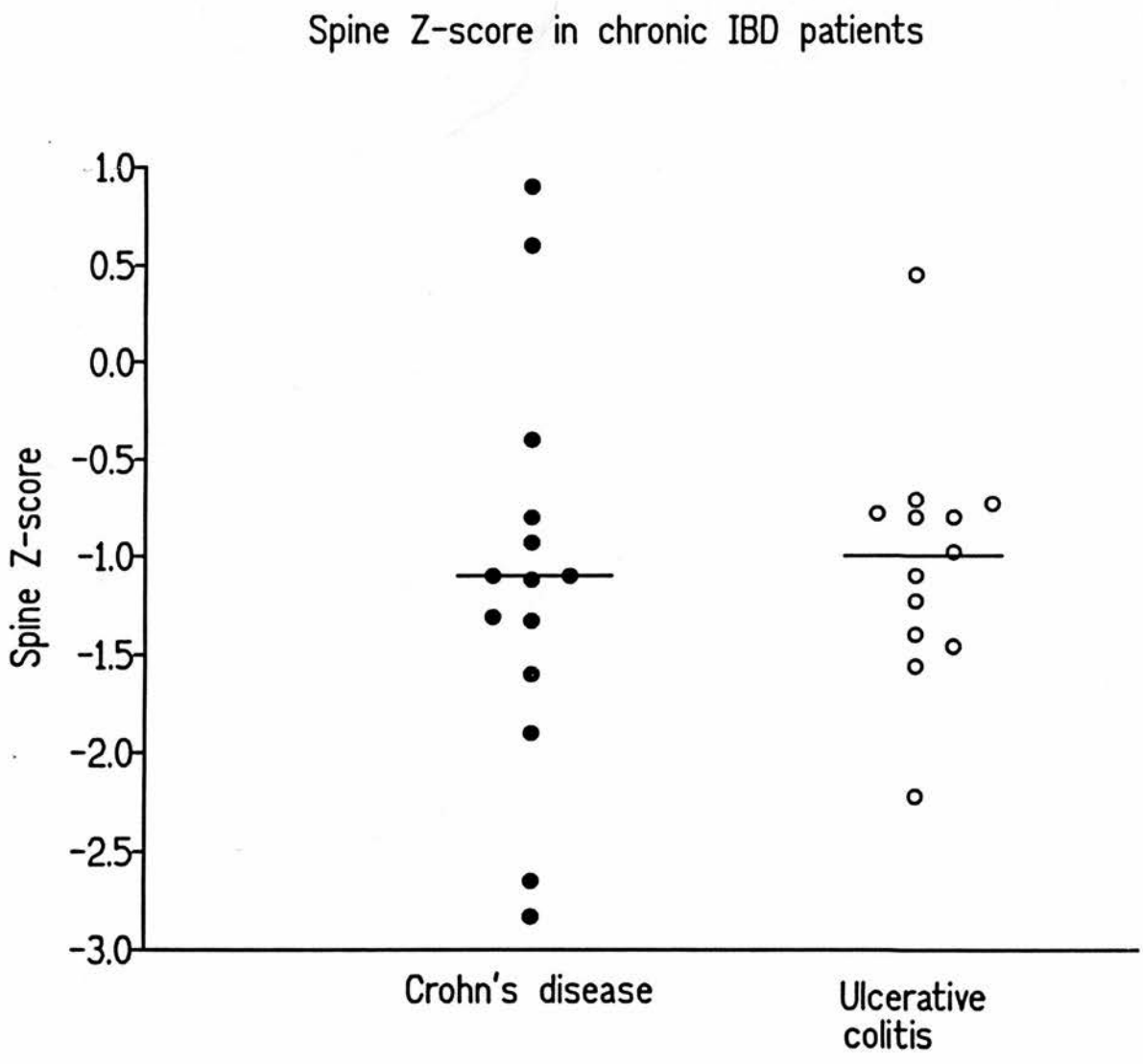

Figure 5.1. Spine bone mineral density Z-scores for patients with long-standing Crohn's disease and ulcerative colitis. The horizontal lines represent mean values.

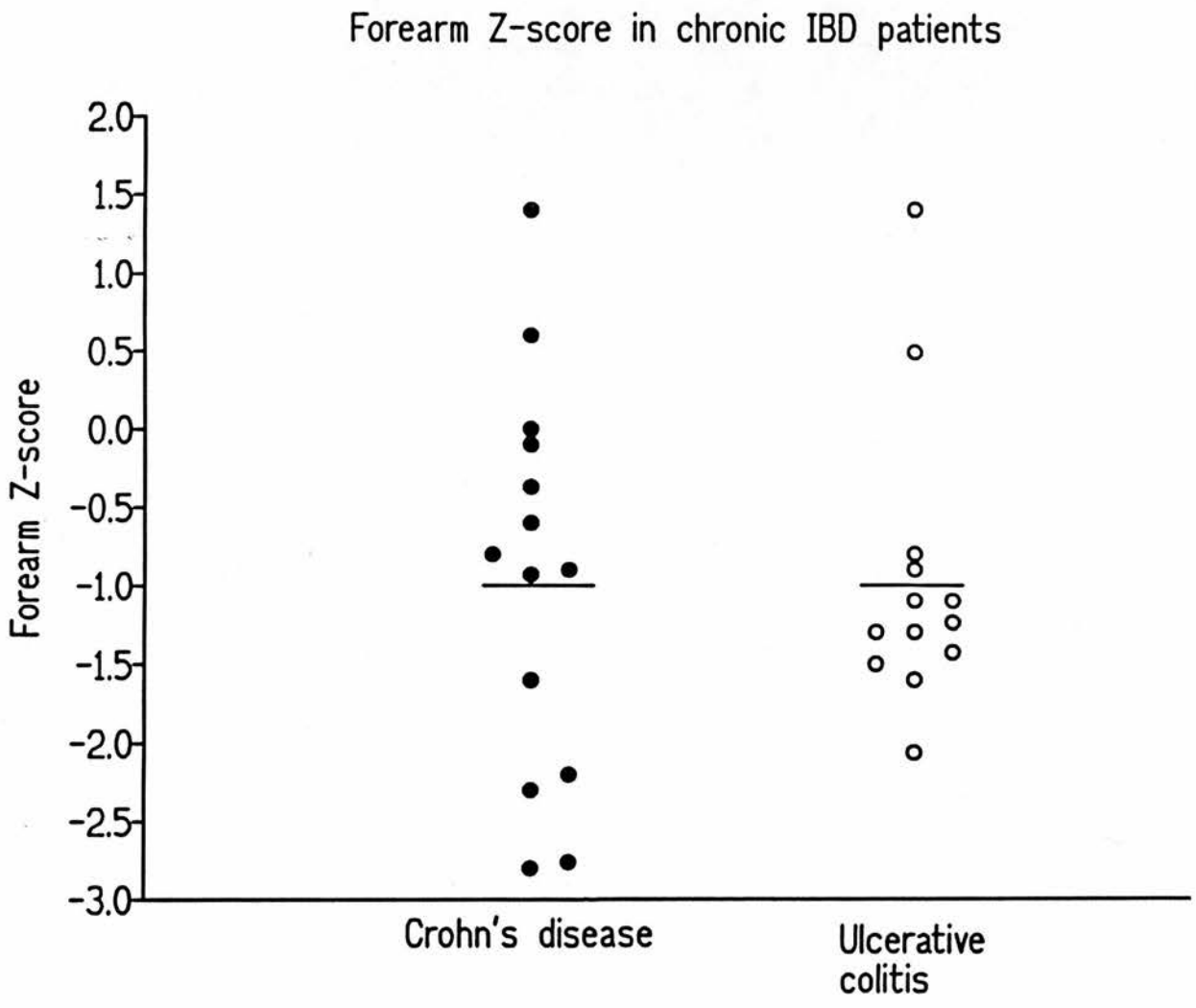

Figure 5.2. Forearm bone mineral density Z-scores in patients with long-standing Crohn's disease and ulcerative colitis. The horizontal lines represent mean values.

5.4.3. Biochemical parameters (Table 5.3)

Plasma calcium, phosphate, alkaline phosphatase levels were normal in both CD and UC. Plasma 25-OH-vitamin D levels were normal in the patients in whom it was measured.

Table 5.3.Biochemical markers of calcium homeostasis

Bone biochemistry	CD mean (SD) n=14	UC mean (SD) n=13
Plasma Calcium (mmol/L)*	2.34 (0.06)	2.36(0.05)
Plasma phosphate (mmol/L)	1.22(0.18)	1.26(0.18)
Alkaline phosphatase (U/L)	84(26)	72(24)
25-OH-D	37(16) (n=10)	45(14) (n=9)

*Total calcium corrected for albumin where appropriate

5.4.4. Effect of macroscopic anatomy of disease

The mean spine and forearm Z-scores of the 7 patients with colonic CD were -0.9 (SD 1.3) and -0.9 (SD 1.1) respectively. This was not significantly different from the mean spine and forearm Z-scores of the 6 patients with small bowel CD (-1.3, SD=1.5 and -1.2, SD=1.4 respectively). In UC, the number of patients with left sided colitis (n=3) were too small for statistical comparison with the group with extensive colitis - spine Z-scores in these 3 patients, were 0.4, -1.0 and -1.2 and forearm Z-scores were 0.4, -1.2 and -1.4.

5.4.5. Effect of disease duration

Disease duration from diagnosis was plotted against spine and forearm Z-scores in both CD and UC. In CD, 14 patients with long-standing disease, 15 newly diagnosed patients (11 of whom had a repeat measurement in 1 year) and 10 patients with disease duration between 1-5 years had spine and forearm Z-scores measured (total no. of patients = 50). There was no correlation between disease duration and spine ($r = -0.2$, p=NS) or forearm ($r = -0.2$, p = NS) BMD Z-scores.

In UC, 13 patients with long-standing disease, 15 newly diagnosed patients (12 of whom had a repeat measurement in 1 year) and 4 patients with disease duration between 1-4 years had spine and forearm Z-scores measured (total no. of patients = 44). Disease duration and BMD Z-scores were correlated significantly (spine $r=-0.5$,$p=0.002$;forearm $r= -0.6$,$p<0.001$).

In CD cumulative steroid dose was not significantly correlated with spine or forearm Z-scores (r= -0.22 and -0.26 respectively); in contrast, in UC cumulative steroid dose was significantly correlated with spine and forearm Z-scores (r = -0.61 and -0.65 respectively; $p<0.01$).

5.5. Conclusions

Unlike at diagnosis, there is no difference in BMD between CD and UC when the disease is long-standing. This is likely to be the explanation why previous studies had failed to detect a difference between CD and UC. BMD is negatively correlated with disease duration in UC, but not in CD. The findings are discussed in chapter X.

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Chapter VI

PATHOGENESIS OF LOW BONE MINERALISATION IN INFLAMMATORY BOWEL DISEASE

6.1. Introduction

A number of biochemical markers of bone metabolism are now available, enabling analysis of osteopenia in terms of bone turnover. Bone mineral density (BMD) is determined by a balance between osteoblast and osteoclast activity. Plasma osteocalcin is a marker of osteoblast function, while urinary pyridinium crosslinks are markers of osteoclast function. Two pyridinium compounds, pyridinoline (Pyd) and deoxy-pyridinoline (Dpd), are maturation products of the lysyl oxidase-mediated crosslinking pathway of collagen (Robins 1983; Ogawa *et al* 1982). Pyd is the major crosslink of cartilage and is prevalent in the collagen of bone and several other tissues, whereas Dpd is primarily located in bone (Robins *et al* 1987; Eyre 1984). There is very little data on whether the osteopenia in IBD is due to increased bone resorption or decreased bone formation. A recent study has described diminished osteocalcin levels suggesting failure of bone formation (Abitbol *et al* 1995). However, increased bone resorption markers have also been described in an abstract (Bjarnason *et al* 1994). After detecting osteopenia in newly diagnosed patients with Crohn's disease (CD), I aimed to recruit a fresh cohort of newly diagnosed patients with IBD to measure these bone turnover markers and compare CD with ulcerative colitis (UC). This study is ongoing, but, in this brief chapter, I will present the results in a small number of patients.

6.2. Subjects and methods

6.2.1. Subjects

Ten newly diagnosed IBD patients were recruited into this study during 1994-95. Six patients (4 males, 2 females, aged 23-61 years, median 29 years)

suffered from CD and 4 (2 males, 2 females, aged 33-51 years, median 38 years) from UC. This was a different cohort from the newly diagnosed patients described in chapter IV. All investigations regarding bone metabolism were done prior to commencement of therapy. None of them had other concurrent illnesses that might affect bones such as primary sclerosing cholangitis or thyroid disorders. None were on calcium, vitamin D supplements or hormone replacement therapy.

6.2.2. Methods

The two pyridinium compounds, Pyd and Dpd were measured in urine. The measurements were carried out in the laboratory of Dr S.P. Robins, Biochemical Sciences Division, Rowett Research Institute, Aberdeen and the method used has been described in chapter III. Creatinine concentration in the urine was also measured and the results expressed as Pyd/creatinine and Dpd/creatinine ratio. The normal adult range of Pyd/creatinine is 15-55 and Dpd/creatinine is 4-15. Previous studies have indicated that there were no marked sex differences nor variations with age in adults (Beardsworth *et al* 1990, Black *et al* 1988, Seibel *et al* 1989). Also, no significant changes in crosslinks excretion were found which were dependent on diet or levels of exercise (Black *et al* 1988). Physiological nyctohemeral variations in the urinary excretion of total pyridinium crosslinks of collagen expressed relative to creatinine are minor in comparison to the changes resulting from arthritic or bone diseases (McLaren *et al* 1993). Hence in this study, a spot morning sample of urine was collected on two consecutive days and stored at -20°C. Blood was collected between 9 and 9.30am and serum separated and stored at -70°C. This was assayed for plasma osteocalcin and bone alkaline phosphatase using the methods described in chapter III at the Rowett Research Institute. The normal adult range for osteocalcin is 2-7 ng/mL and for bone alkaline phosphatase is 10-23 U/L. Plasma calcium, albumin and alkaline phosphatase were measured. BMD was measured at spine and forearm by DEXA, and the sites of measurement were as described in chapter III. Blood and urine collection were done on the same day as the DEXA scan and the patient returned within two days with another urine sample. The patients were asked to keep a CDAI diary card for a week prior to their visit for the scan.

6.2.3. Statistical analysis

Student's t-test was used to compare results between CD and UC.

6.2.4. Ethical considerations

The study was approved by the Medicine Subcommittee of the Lothian Area Ethics of Research Committee. Each patient gave informed verbal consent.

6.3. Results

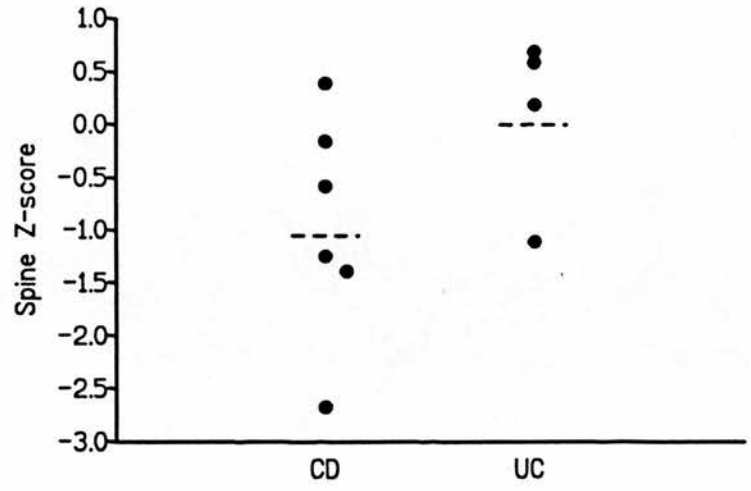
Though the number of patients were small, the results again confirm low BMD in newly diagnosed patients with CD compared with UC (figure 6.1). The mean spine Z-score in patients with CD was -0.94 (SD 1.1) and in UC 0.1 (SD 0.83), but the difference did not reach statistical significance. The mean forearm Z-score was -0.68 (SD 1.0) in patients with CD, and this was significantly lower ($p<0.05$) than that in UC (mean 0.50; SD 0.28).

Figures 6.2 and 6.3 show the results of urinary pyridinium crosslinks, plasma osteocalcin and bone alkaline phosphatase in patients with CD and UC. Mean urinary Pyd/creatinine was 75.5 (SD 66.6) in patients with CD and 36.2 (SD 13.7) in patients with UC (p=NS). Mean urinary Dpd/creatinine was 19.5 (SD 15.3) in patients with CD and 10.9 (SD 2.5) in patients with UC (p=NS). Urinary Pyd/creatinine and Dpd/creatinine were significantly correlated ($r=0.96$; $p<0.0001$). Three out of 6 patients with CD, but none with UC had pyridinium crosslinks excretion in the urine higher than the upper limit of normal. Mean plasma osteocalcin was not different in CD compared with UC (3.2 vs 2.6 ng/mL). Mean plasma bone alkaline phosphatase too was not different (19.8 vs 20.2 U/L). Bone formation markers were within or close to the normal range in all patients.

Urinary pyridinium crosslinks were not correlated with either spine or forearm Z-scores. Plasma calcium and alkaline phosphatase were normal in all patients.

6.4. Conclusion

This is a small study and hence conclusions drawn from it must be treated with caution. It confirms low bone mineral density in newly diagnosed patients with CD. Biochemical data consistent with bone resorption were found in 3 out of 6



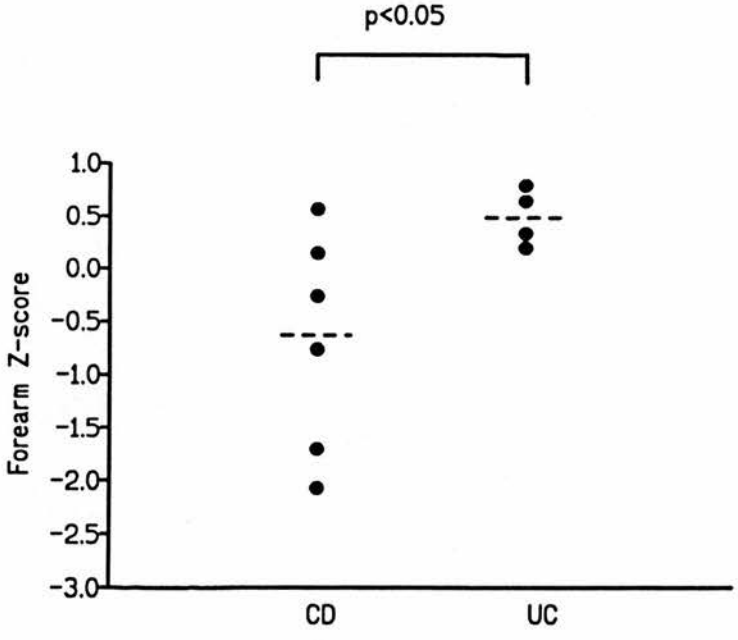


Figure 6.1. Spine and forearm BMD values in newly diagnosed patients with CD and UC in whom metabolic markers of bone metabolism were measured. The horizontal lines represent mean Z-scores.

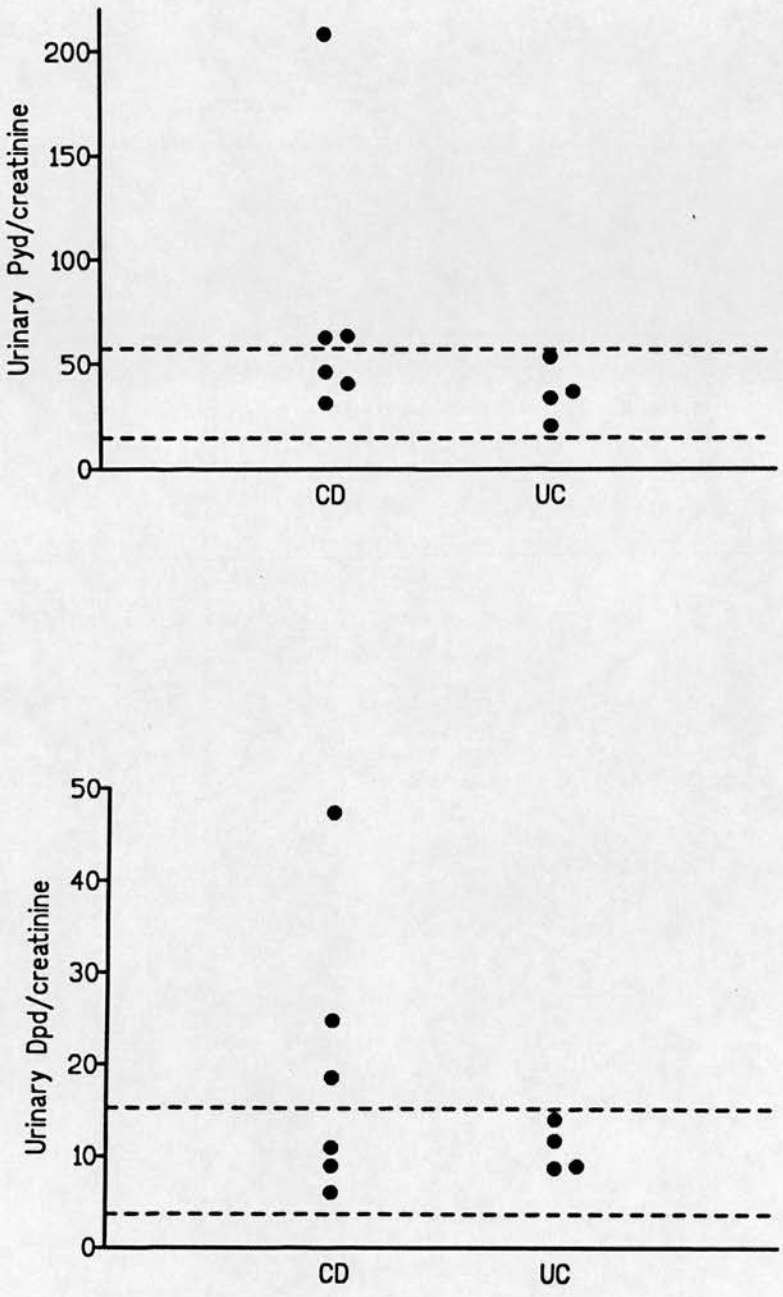


Figure 6.2. Bone resorption markers, Urinary Pyd/creatinine and urinary Dpd/creatinine in patients with CD and UC. The horizontal lines represent normal ranges

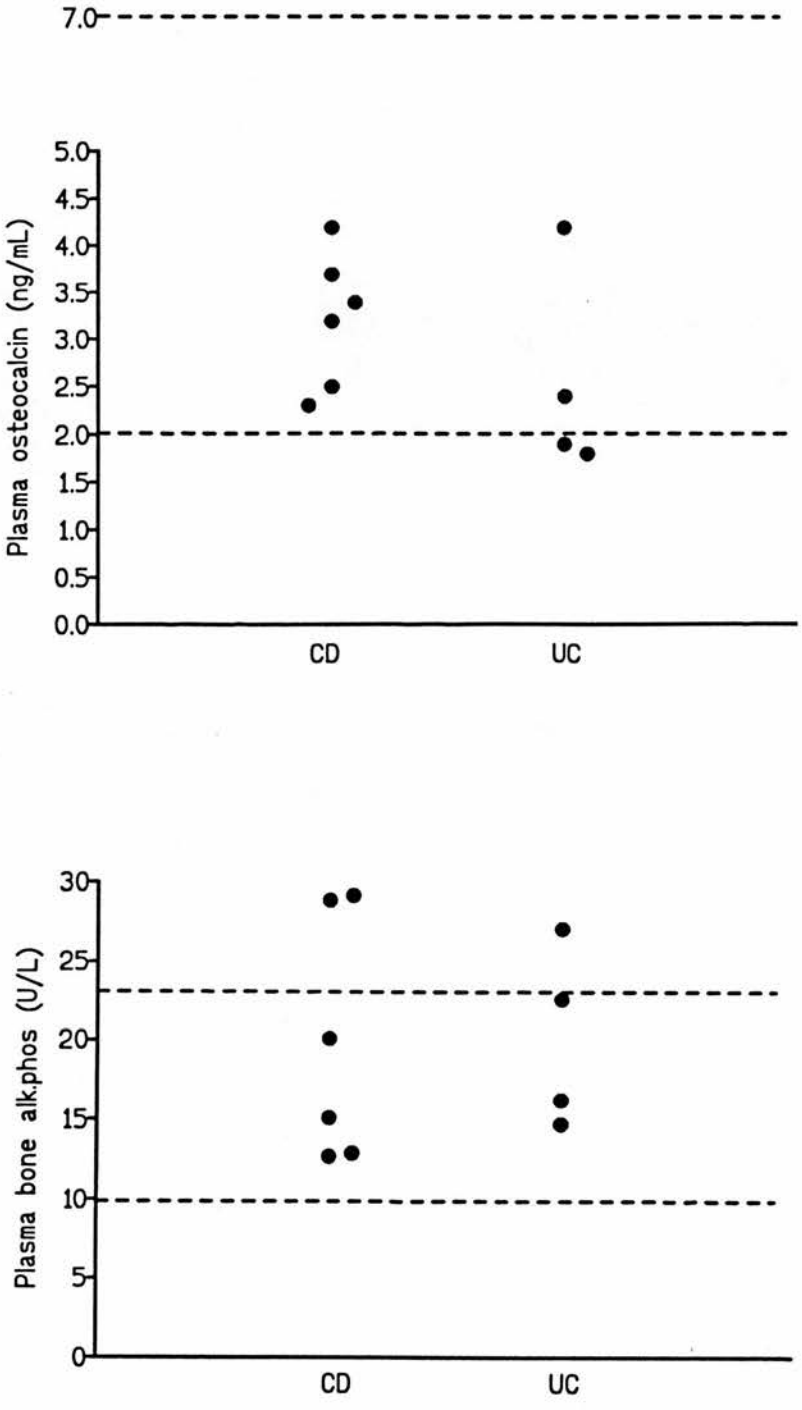


Figure 6.3. Bone formation markers, plasma osteocalcin and plasma bone alkaline phosphatase in patients with CD and UC. The horizontal lines represent normal ranges.

patients with CD but in none of the 4 with UC. Bone formation markers were generally within or close to normal limits. Owing to the small number of patients studied, no firm conclusions can however be reached.

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Chapter VII

BODY COMPOSITION AT THE BEDSIDE

7.1. Introduction

Appraisal of nutritional status is still a frequently neglected component of clinical examination (Barton *et al* 1989). In many situations it should be mandatory to measure body weight accurately, or, alternatively, to calculate the body mass index (BMI), which takes account of appropriateness of weight for height, and allows patients and healthy individuals to be categorised as underweight, normal (in the range 20-25 kg/m^2), or overweight (Owen 1988).

For any more sophisticated analysis of body composition, it is necessary to differentiate in some way between body fat and fat-free or lean body mass (LBM). This can, for example, be approached by densitometry - direct measurement of body specific gravity by underwater weighing - a method used by physiologists but unsuitable for clinical practice. In many settings, body fat content is calculated from multiple measurements of skinfold thickness, an apparently simple and inexpensive approach. However the assumptions underlying this method - that subcutaneous fat reflects total body fat, and that the chosen site of measurement of subcutaneous fat represents its average thickness - are both incorrect (Lukaski 1987, Heymsfield *et al* 1979). In recent years several different body composition analysis methods have been developed for research purposes, including neutron activation analysis, ^{40}K counting and dual energy X-ray absorptiometry (DEXA). All give broadly comparable results, despite their very different theoretical bases, and at present, there is no *a priori* reason for choosing any one technique as the gold standard (Lukaski 1987). In my institution, DEXA has proved to be a precise and reproducible method of measuring lean body mass (Hannan *et al* 1993).

Recently, medical physicists have exploited the fact that electrical conductivity is much greater in lean tissues than in fat, and have built machines that measure the bioelectrical impedance of the body; from this information the lean:fat ratio can be derived. User-friendly versions of these machines have found a place in sports medicine and nutrition/slimming clinics but are rarely if ever used in clinical practice, partly because doctors do not appreciate that the technique is entirely valid scientifically. I have evaluated the accuracy and ease of use of one

of these small, hand-held, menu driven, bioelectrical impedance analysis machines. The present account also illustrates the useful information that this simple instrument can provide on the body composition of adult patients with gastro-intestinal or nutritional diseases.

7.2. Subjects and methods

7.2.1. Subjects

One hundred and fourteen subjects were recruited for a technical comparison of various body composition measurement methods. Fifty-eight (2 males; 56 females) were healthy teenage volunteers(11-13 years;median 12 years) taking part in an ongoing growth study based on DEXA and bioelectrical impedance plethysmography. Measurement of bioelectrical impedance by the *hand held* machine was added to the protocol. The remaining 56 subjects were adults. They were: (a) 17 patients with eating disorders (all female; median age 26 years, range 16-44 years; one grossly obese and 16 underweight); (b) 7 patients with chronic pancreatitis (6 males, 1 female; median age 47 years, range 33-58 years). These two groups were participating in ongoing studies of body composition based on DEXA and *in-house* bioelectrical impedance plethysmography and measurement by the *hand-held* machine was added to the protocol. (c) 14 healthy volunteers (hospital staff) (5 males, 9 females; median age 32 years, range 20-43 years); (d) 18 patients with inflammatory bowel disease (IBD) (9 males, 9 females; median age 34 years, range 21-64 years). Fourteen patients had Crohn's disease (CD) (active disease in 3 patients) and 4 patients had ulcerative colitis (UC) (1 had active disease).

7.2.2. Methods

The subjects were weighed and their height measured. LBM was measured by two bioelectrical impedance machines and by DEXA. The details of the machines and methods are described in chapter III. All measurements were made on the same day in the Medical Physics Department. Subjects were not specifically asked to fast.

7.2.3. Statistical analysis

Pearson's correlation coefficient was used to examine relationships between the measurements by different methods. Comparison between LBM measured by the DEXA method and *in house* bioelectrical analysis method was also performed by the Bland and Altman method which plots the paired difference between LBM measured by the two methods against the mean of these two readings. The mean difference (d) provides an estimate of the bias, and the limits of agreement is given by the interval d-2s to d+2s, where s is the standard deviation of d (Bland and Altman 1986).

7.3. Results

Trained Medical Physics personnel are required to operate the DEXA and the *in-house* bioelectrical impedance machine (RJL systems), and to calculate the results. With DEXA, imaging time was typically 12 minutes and analysis time 14 minutes. Bioelectrical impedance analysis using the *in-house* machine typically took about 10 minutes to perform and 10 minutes to analyse.

Doctors learned to use the *hand-held*, menu-driven machine after about 30 minutes reading of the instruction manual. The machine was easily operated by nursing staff after 10 minutes of verbal instruction and demonstration. It typically took 8 minutes to make the measurements; the machine displays the results immediately. The coefficient of variation for repeated measurements of electrical impedance was 0.4% when measured by the *in-house* bioelectrical impedance analyser and 0.7% when measured by the *hand-held* machine.

7.3.1. Technical comparison of the in-house Vs hand-held bioelectrical impedance analyser

In the entire series of 114 subjects, impedance results measured by the *hand-held* machine correlated very closely with results from the *in-house* machine, both resistance ($r=0.996$; $p<0.0001$; Figure 7.1), and impedance ($r=0.996$; $p<0.0001$). The regression equations show that the two methods give virtually identical results.

7.3.2. Comparison of lean body mass measured by DEXA with results from the hand-held bioelectrical impedance analyser

It is likely that the prediction equation for LBM from impedance will be different in children and adults. As there are as yet no validated equations for children, I confined comparison of calculated LBM to the data from adult subjects. Figure 7.2 shows the lean body mass (kg) measured by DEXA plotted against that measured by the *hand-held* machine in the 56 adult subjects. The two measurements were significantly correlated ($r=0.960$; $p<0.0001$). The values were virtually identical over the range of lean body mass - the regression equation was $y = 3.36 + 0.952x$. This also held true for the individual patient groups (Healthy adult volunteers $r=0.970$; patients with an eating disorder $r=0.974$; chronic pancreatitis group $r=0.979$; IBD group $r=0.937$).

In the eating disorder patients, the prediction equation previously described was also used to calculate the lean body mass directly from the impedance values - this gave an r value of 0.959 against DEXA measurements. In IBD patients, the prediction equation derived by Jeejeebhoy's group was also used to calculate the lean body mass from impedance values - this gave an r value of 0.904 against DEXA measurements.

The agreement between the two methods of measuring BMD was also analysed by the Bland and Altman method. There was no correlation ($r=0.1$; p=NS) between the difference and the mean of the paired observations. The bias was 2.6% and the limits of agreement -9.2% to 14.4%.

7.3.3. Relationship of BMI with body composition

BMI, which is an index of over- or under-weight, is, not unreasonably, often also used to infer that patients are over- or under-nourished. I investigated the relationship between BMI and body composition, using lean body mass expressed as percentage lean (LBM X 100/body wt). For the whole group of 56 adult subjects, BMI correlated weakly and inversely with percentage lean as measured using DEXA ($r=-0.573$, $p<0.0001$) and using the *hand-held* machine ($r=-238$,p=NS). There was a better correlation between BMI and lean body mass (kg)/height(m)^2 measured using DEXA ($r=0.862$,$p<0.0001$) and using the *hand-held* machine ($r=0.857$,$p<0.0001$). This is of course, identical to the relationship between lean body mass and total body weight.

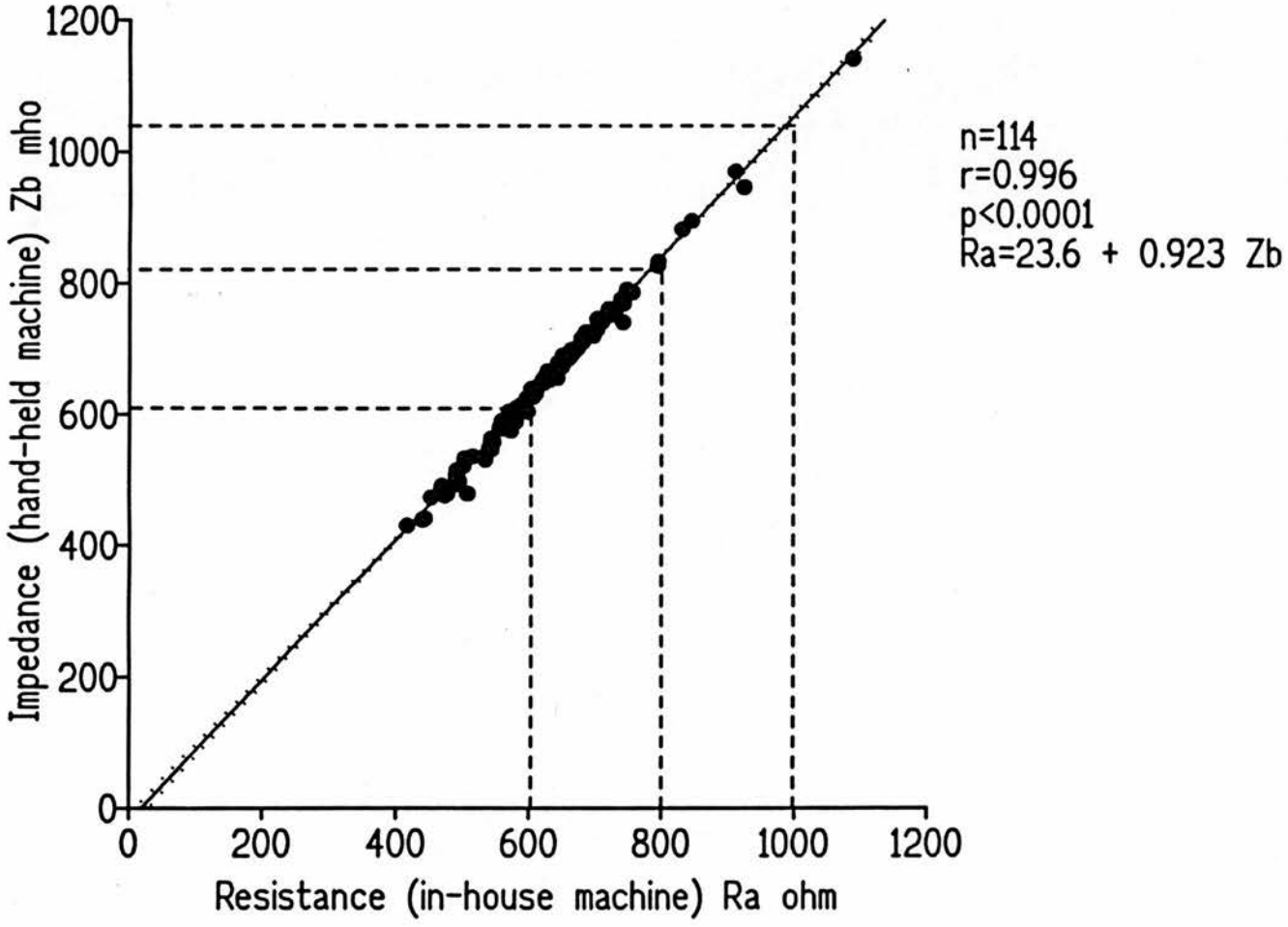

Figure 7.1. Correlation between resistance measured by the *in-house* bioelectrical impedance machine and impedance measured by the *hand-held* bioelectrical impedance machine (n=114). The dotted line on either side of the regression line represent 95% confidence intervals. Dashed vertical and horizontal lines of equivalence are shown.

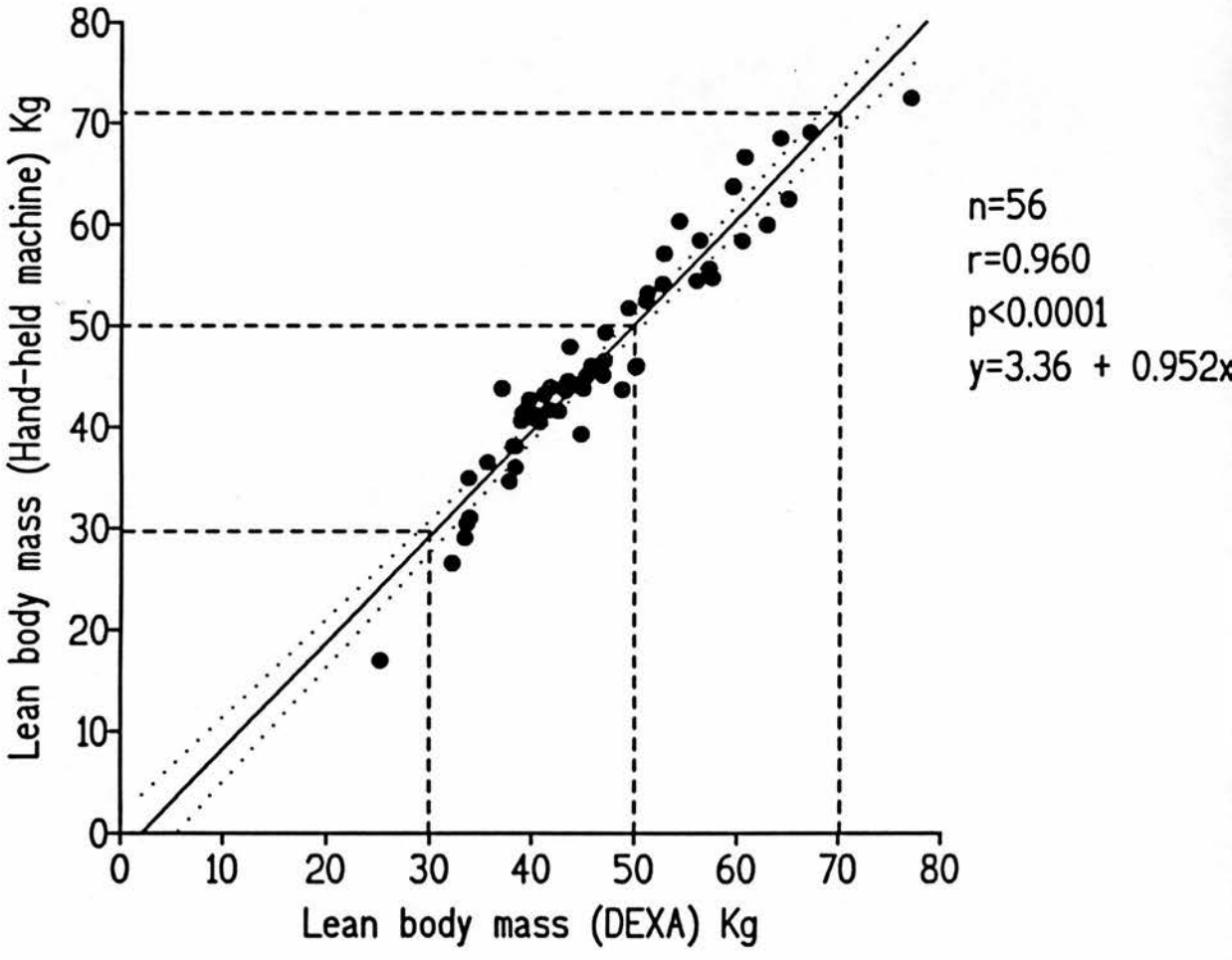

Figure 7.2. Correlation between LBM in Kg measured by DEXA and the *hand-held* bioelectrical impedance machine in the 56 adults. Dotted line on either side of the regression line represents 95% confidence interval. dashed vertical and horizontal lines of equivalence are shown.

7.3.4. Information obtained from consideration of percentage lean in relation to BMI

Table 7.1 shows the lean body mass, percent lean, BMI and body weight in the groups of adult subjects. Percent fat can readily be calculated (i.e. 100 - percent lean). Relationships between percent lean and BMI in males and females are shown in figures 7.3 and 7.4. Those with BMI < 20 were classified as underweight. The 5 healthy men had a BMI range of 23.2-26.5 and their percent fat (i.e. 100-percent lean) ranged from 14.2-20.8. Seven of the 9 healthy women had a BMI > 20 (range 21.3-27.6) and their percent fat ranged from 26.8 - 36.1. For these similar BMI ranges, two-compartment body composition figures for men and women are significantly different ($p<0.01$). Two healthy women had BMI < 20 (19.4,19.7) and their percent fat values were 24.1 and 29.1 respectively. Results for the chronic pancreatitis patients show generally normal BMI values but a lower proportion of lean than in the healthy subjects in the same BMI range. Three of the 9 men and four of the 9 women with IBD were underweight and five of these (two men, three women) had lower percent fat than the normal men. In contrast, the patients with IBD who had BMI > 20 had a similar percent fat to the normal volunteers. This suggests that underweight IBD patients (none of whom was acutely ill) were depleted of fat rather than lean. The percent fat in the 13 underweight eating disorder women had a much wider spread (11.2-41.3) indicating that some had lost both fat and lean tissue. One of this group, with a BMI of 11, 58.1% lean, 41.3% fat was clinically marasmic.

7.3.5. Monitoring of clinical progress in undernourished patients

When severely undernourished patients receive intensive nutritional support, progress is generally monitored by changes in body weight and in biochemical parameters such as serum albumin. Analysis of body composition has rarely been used, as most of these patients are very ill and methods such as neutron activation analysis or DEXA are impractical. A portable, menu driven machine can, however, be used in this situation, if fluid balance is stable and the

patient is mobile enough to be weighed. Figure 7.5 presents data for a patient with complicated CD.

Table 7.1. Lean Body Mass measured by the *hand-held* bioelectrical impedance machine in different diagnostic groups.

Subject group (n)	Lean Body mass Kg. Mean (SD, range)	%Lean Body Mass. Mean (SD, range)	Body mass index Kg/m^2 Mean (SD, range)	Body weight Kg. Mean (SD,range)
Healthy adult volunteers				
Men(n=5)	62.8 (7.2) (54.4-69.1)	81.5 (2.9) (79.2-85.8)	25.0 (1.5) (23.2-26.6)	77.1 (8.1) (67.9-86.5)
Women(n=9)	41.9 (5.5) (31.0-51.7)	69.52 (3.9) (63.9-75.9)	23.3 (3.0) (19.4-27.7)	60.5 (9.3) (43.7-79.0)
Women with eating disorder				
Undernourished (n=16)	37.2 (8.1) (17.0-46.0)	76.5 (7.0) (58.6-88.8)	17.6 (2.9) (11.0-21.5)	48.3 (8.5) (29-59)
Overweight (n=1)	49.3	62.9	30.3	78.4
Chronic pancreatitis				
Men(6)	57.2 (10.8) (43.7-72.5)	74.0 (3.5) (70.7-80)	25.5 (5.4) (19.1-32.7)	77.5 (15.6) (57.8-101.3)
Woman(1)	38.1	65.8	21.5	57.9
IBD				
Men(n=9)	55.4 (5.3) (45.1-63.7)	84.6 (6.7) (78.9-90.3)	22.7 (3.6) (17.9-27.4)	66.6 (8.3) (54.0-78.8)
Women(n=9)	43.0(3.6) (36.5-47.9)	73.8 (9.7) (60.0-90.9)	21.7 (3.9) (17.6-29.2)	59.3 (10.4) (48.2-77.5)

7.4. Conclusion

It is increasingly important that medical and nursing staff understand and use the simple principles of anthropometry, including the two compartment model of body composition. My experience shows that a portable bioelectrical impedance machine can be introduced easily into clinical practice. At present there are no standard population-based tables on the percent lean values related to age, sex, stature, or body frame, but with a simple machine such as that which I have used, these data could be collected in future population studies.

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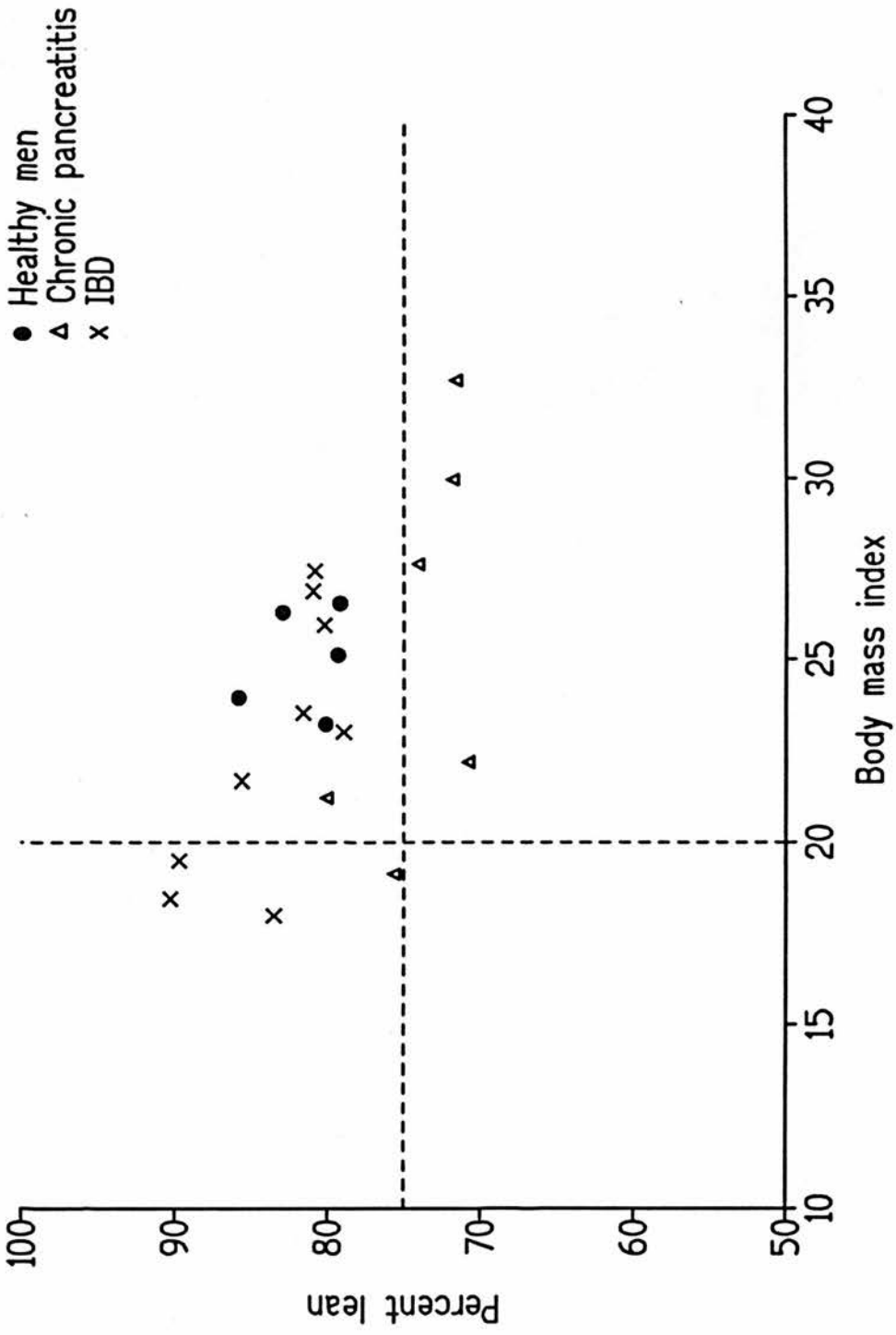

Figure 7.3. Percent lean in different groups of adults plotted against their BMI in male patients. BMI less than 20 is considered underweight. A horizontal line is drawn through 75% lean for reference.

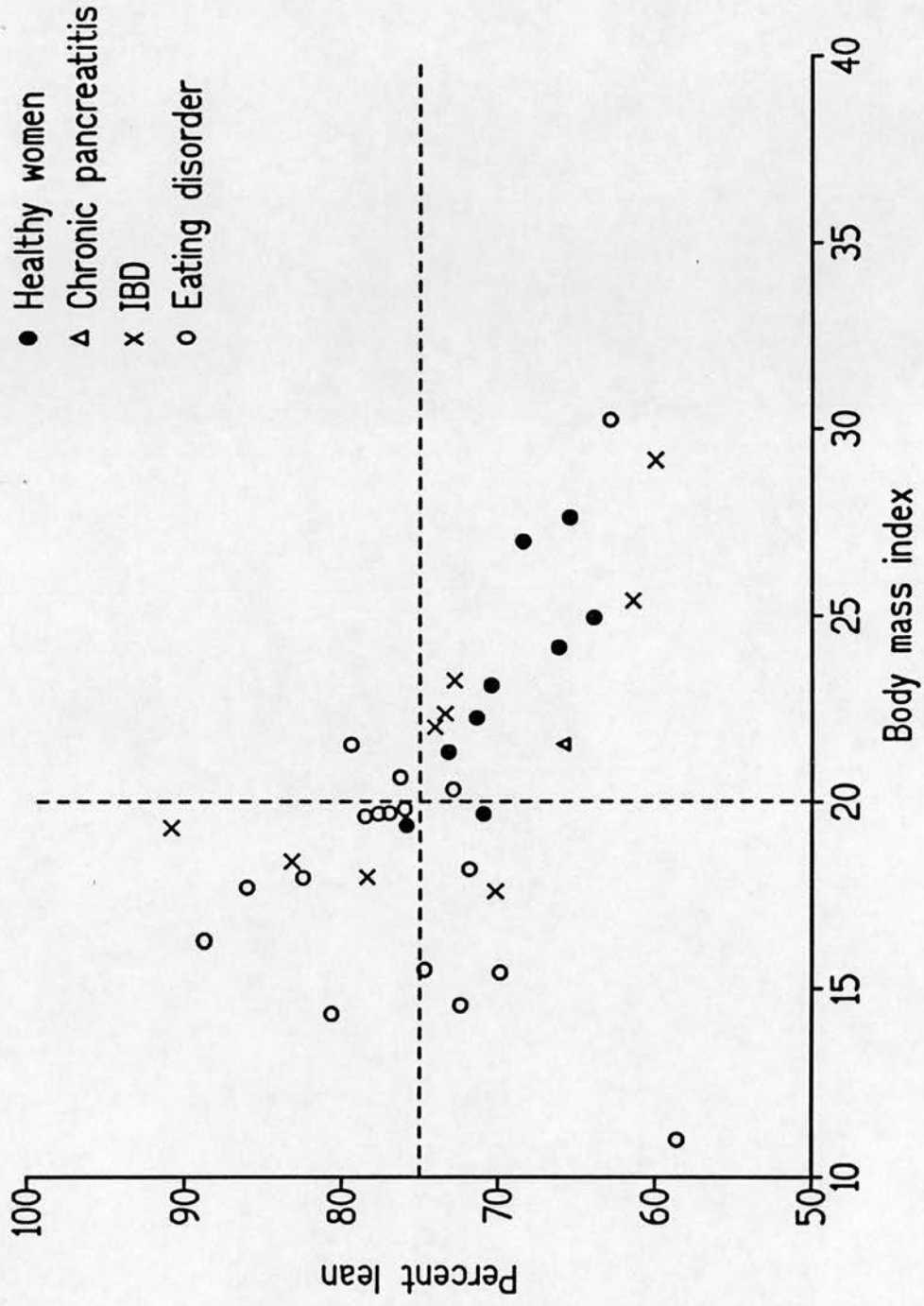

Figure 7.4. Percent lean in different groups of adults plotted against their BMI in female patients. BMI less than 20 is considered underweight. A horizontal line is drawn through 75% lean for reference.

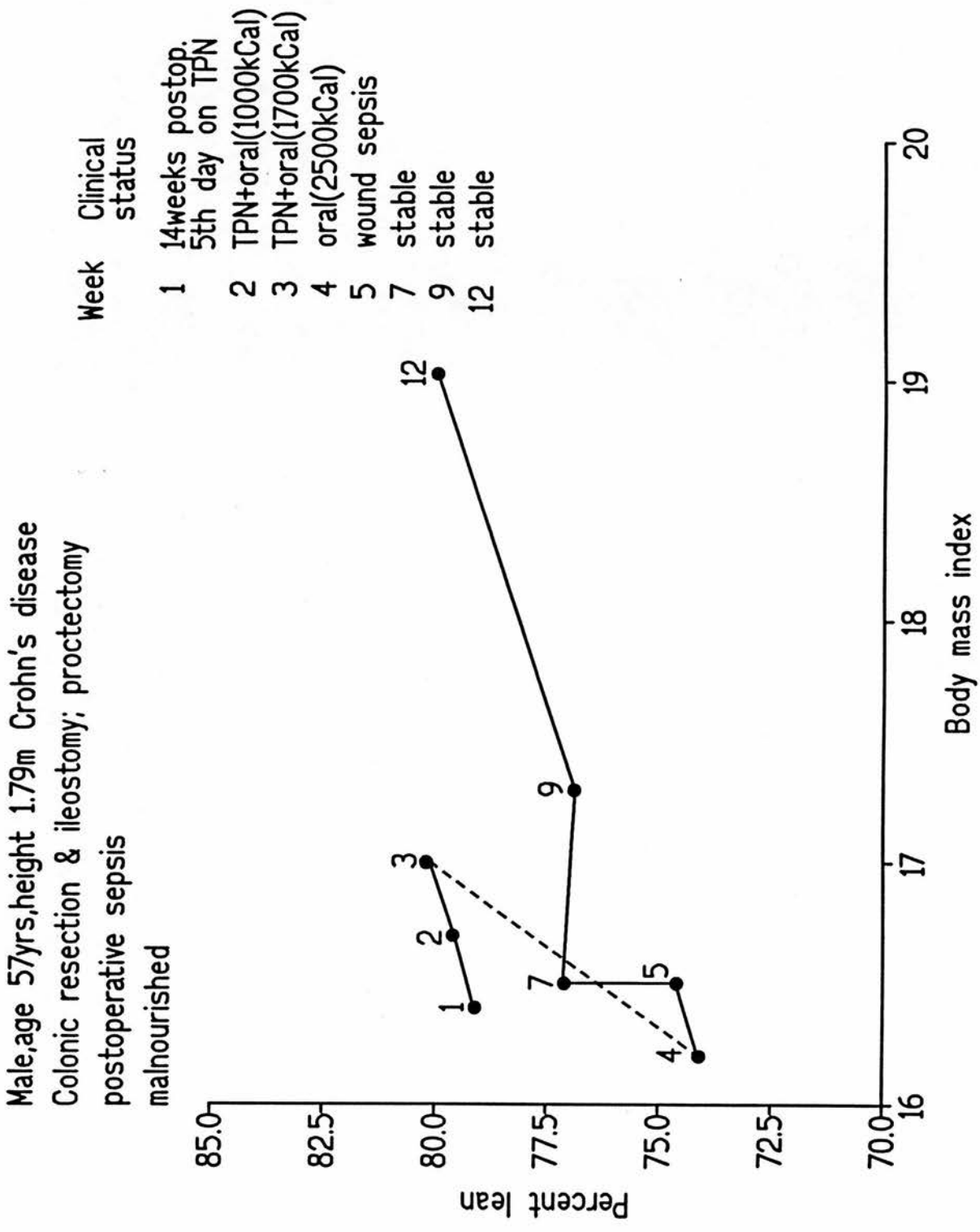

Figure 7.5. Serial measurements of percent lean by the *hand-held* machine and BMI in a malnourished 57-year-old man with complicated Crohn's disease after commencement of total parenteral nutrition.

Chapter VIII

BODY COMPOSITION IN PATIENTS WITH INFLAMMATORY BOWEL DISEASE AND RELATIONSHIP TO BONE MINERAL DENSITY

8.1 Introduction

Bone is part of the lean compartment as discussed in chapter II. Hence, it was of interest to determine the relationship between bone mineral density (BMD) and lean body mass in patients with Crohn's disease (CD) and ulcerative colitis (UC). In other words, the aim was to investigate whether changes in body composition occur in association with low BMD.

8.2. Patients and methods

Thirtythree patients with inflammatory bowel disease were recruited from the outpatients clinic for both measurement of body composition and BMD simultaneously. These patients were derived from cohorts already described in chapters V and VI. Twenty-three of these patients had CD and 10 patients had UC. The patient characteristics are shown in table 8.1.

Table 8.1. Patient characteristics

	Crohn's disease	Ulcerative colitis
n	23	10
Median age (range)	33 (22-71)	43 (22-61)
Sex M:F	13:10	3:7
Median disease duration (range)	5 (0-21) years	4 (0-16) years
Median BMI (range)	21 (16-27)	23 (18-38)
Duration of steroid therapy	18 (0- 84) months	16 (0-76)

Spine and forearm Z-scores were compared with percent lean, so that both male and female patients could be pooled together and compared. BMD and body composition were measured by DEXA at the same time. The details of the methods

are given in chapter III. Accurate height and weight were recorded and body mass index (BMI) was calculated.

8.2.1. Statistical analysis

Student's t-test and Pearson's correlation test were used for statistical analysis.

8.2.2. Ethical considerations

The study was approved by the Medicine Subcommittee of the Lothian Area Ethics of Research Committee. Each patient gave informed verbal consent.

8.3. Results

Figures 8.1 and 8.2 show the BMI and percent lean of patients with CD and UC separated by sex. As is anticipated, male IBD patients had a significantly higher percent lean (mean 83.0; SD 5.8) compared with female IBD patients (mean 70.7; SD 6.7; $p<0.0001$). For both male and female patients, the percent lean increases (i.e. the fat percent falls) as the BMIs decrease. Table 8.2 shows the percent lean results for CD and UC patients by sex.

Table 8.2. Percent lean in male and female IBD patients by sex.

	Mean percent lean	SD	Range	p
Male				
Crohn's disease (n=13)	81.1	6.4	70.9 - 88.8	NS
Ulcerative colitis (n=3)	81.6	2.6	78.6 - 83.1	
Female				
Crohn's disease (n=10)	72.1	5.5	63.1 - 78.9	NS
Ulcerative colitis (n=7)	67.7	7.7	55.1 - 78.5	

Figures 8.3 and 8.4 show spine and forearm BMD Z-scores plotted against percent lean in patients with CD. There is a significant inverse correlation of spine ($r=-0.59$, $p=0.003$) and forearm ($r=-0.44$, $p<0.05$) Z-scores with percent lean. In other words, osteopenia was associated with increased (and not decreased)

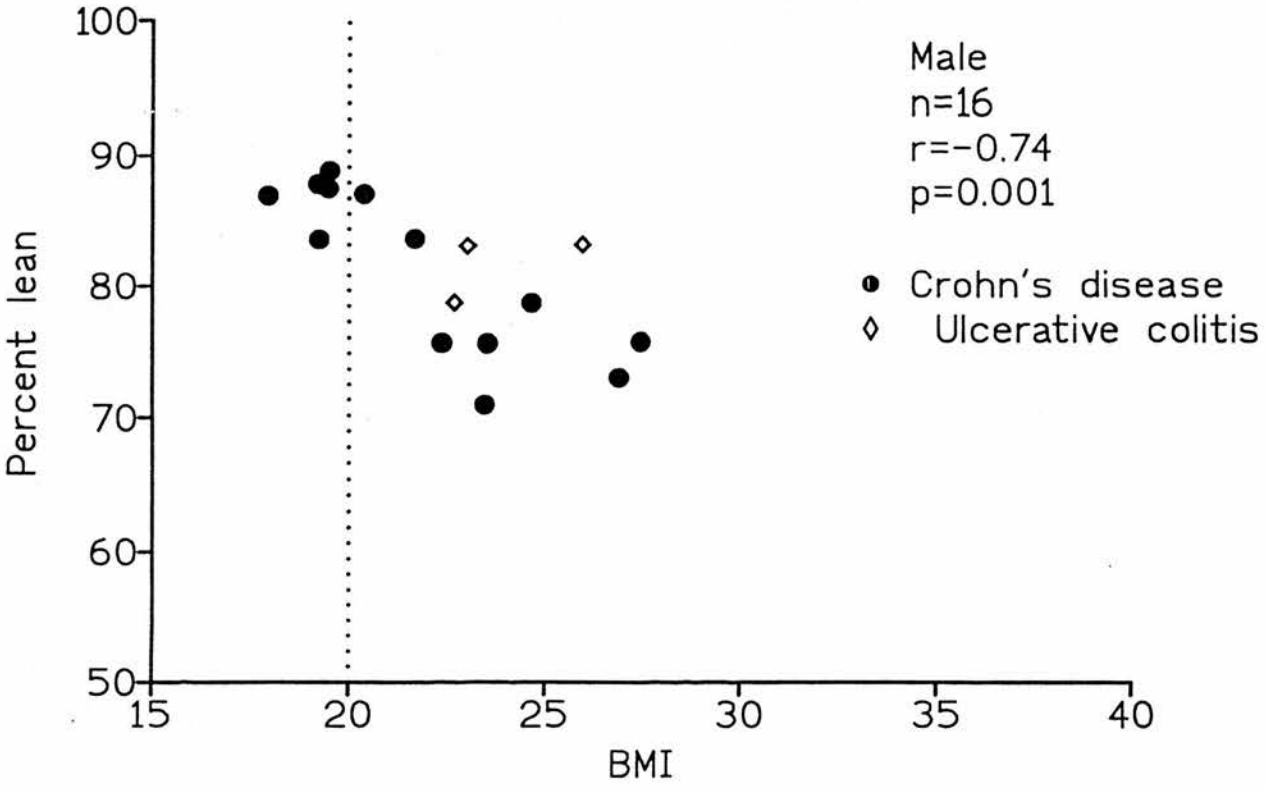

Figure 8.1. Body mass index (BMI) Vs percent lean in male IBD patients. BMI < 20 is considered underweight and all underweight patients suffered from Crohn's disease.

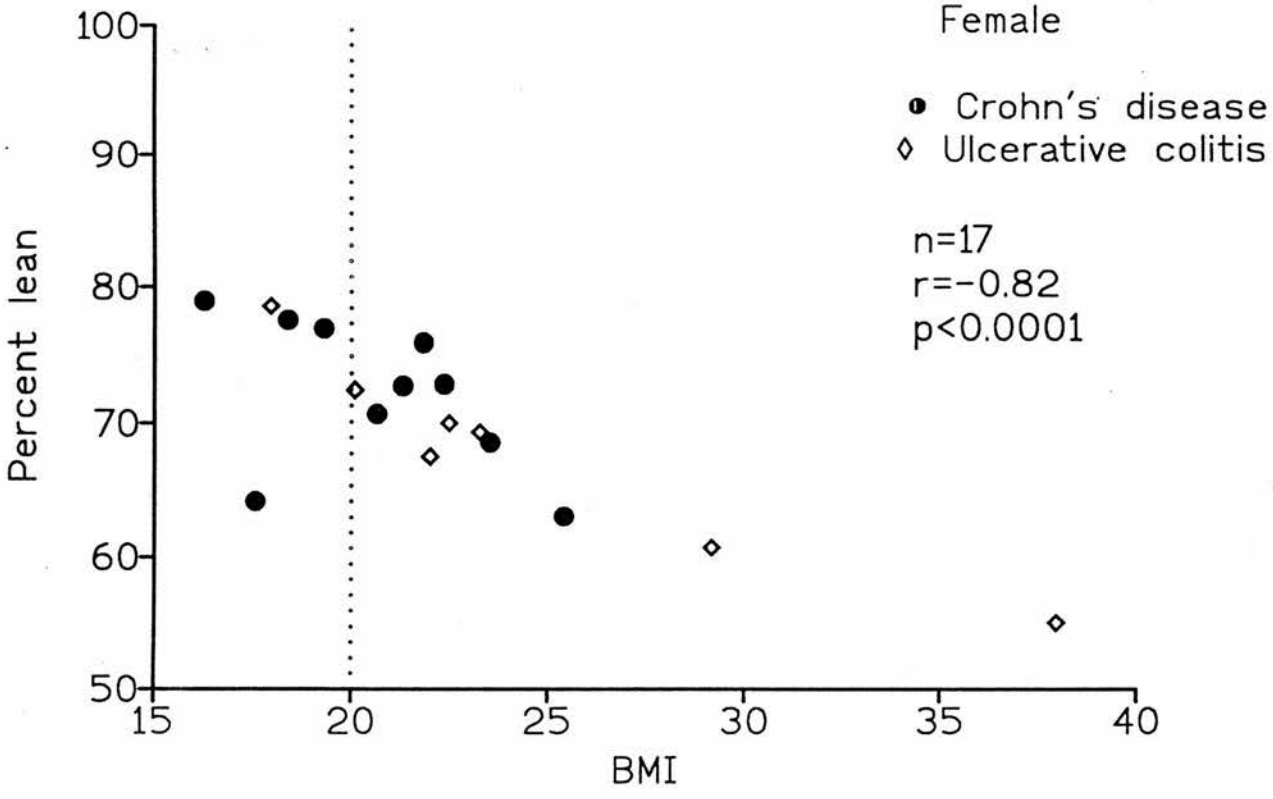

Figure 8.2. Body mass index (BMI) Vs percent lean in female IBD patients. BMI < 20 is considered underweight and, all but one underweight patients suffered from Crohn's disease. The only underweight ulcerative colitis patient was known to be anorectic.

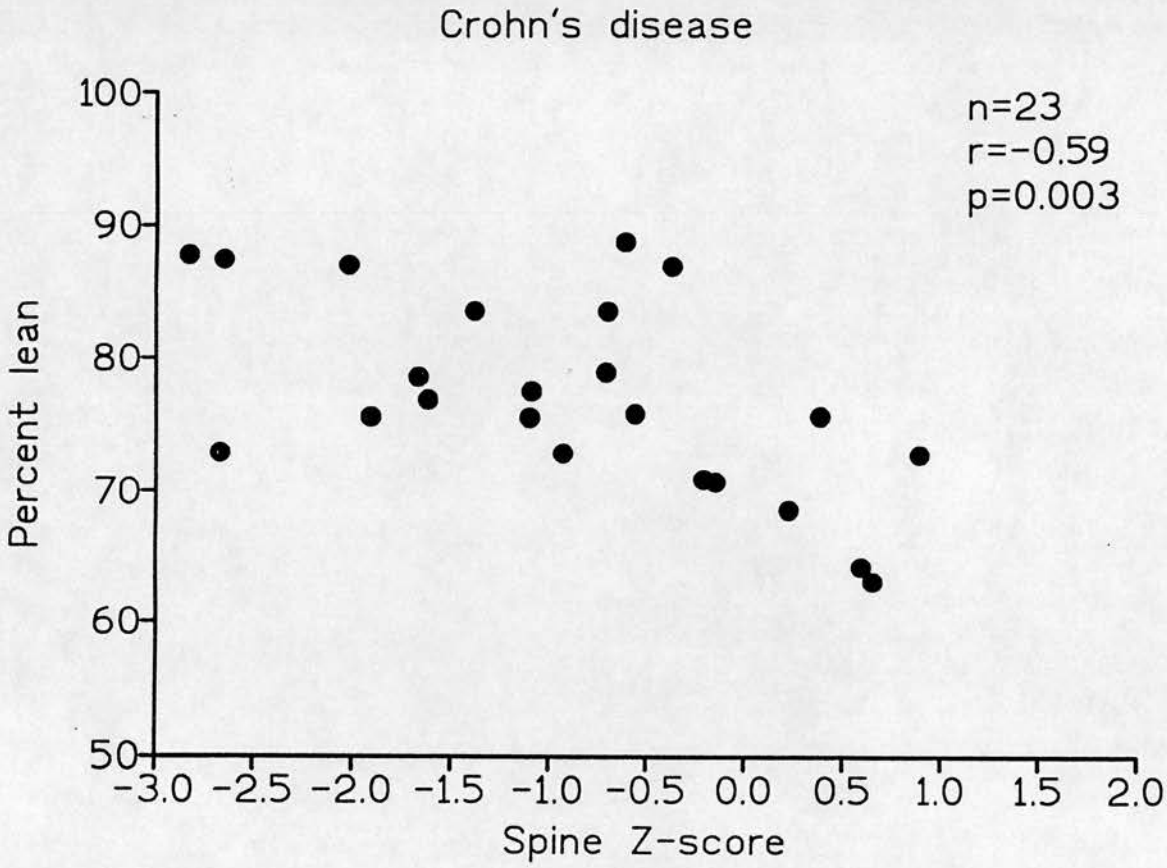

Figure 8.3. Spine Z-scores plotted against percent lean in patients with Crohn's disease.

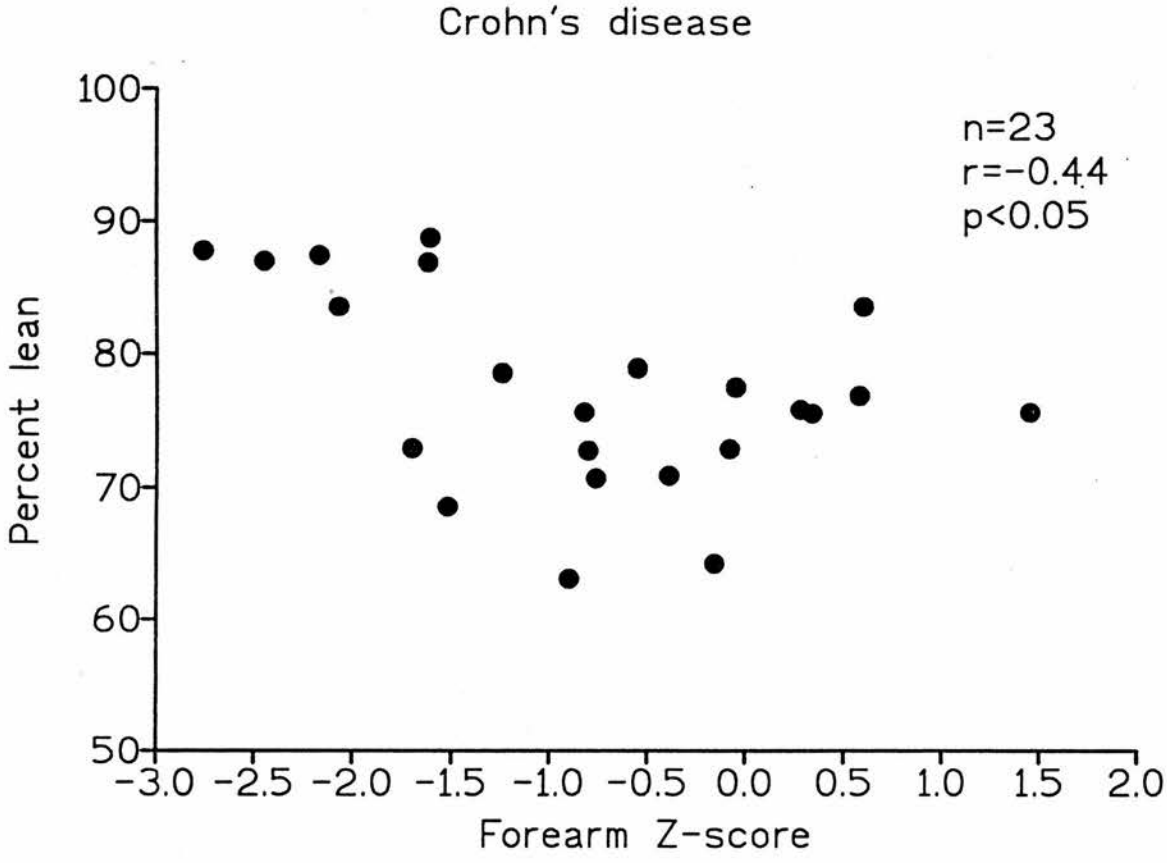

Figure 8.4. Forearm Z-scores plotted against percent lean in patients with Crohn's disease.

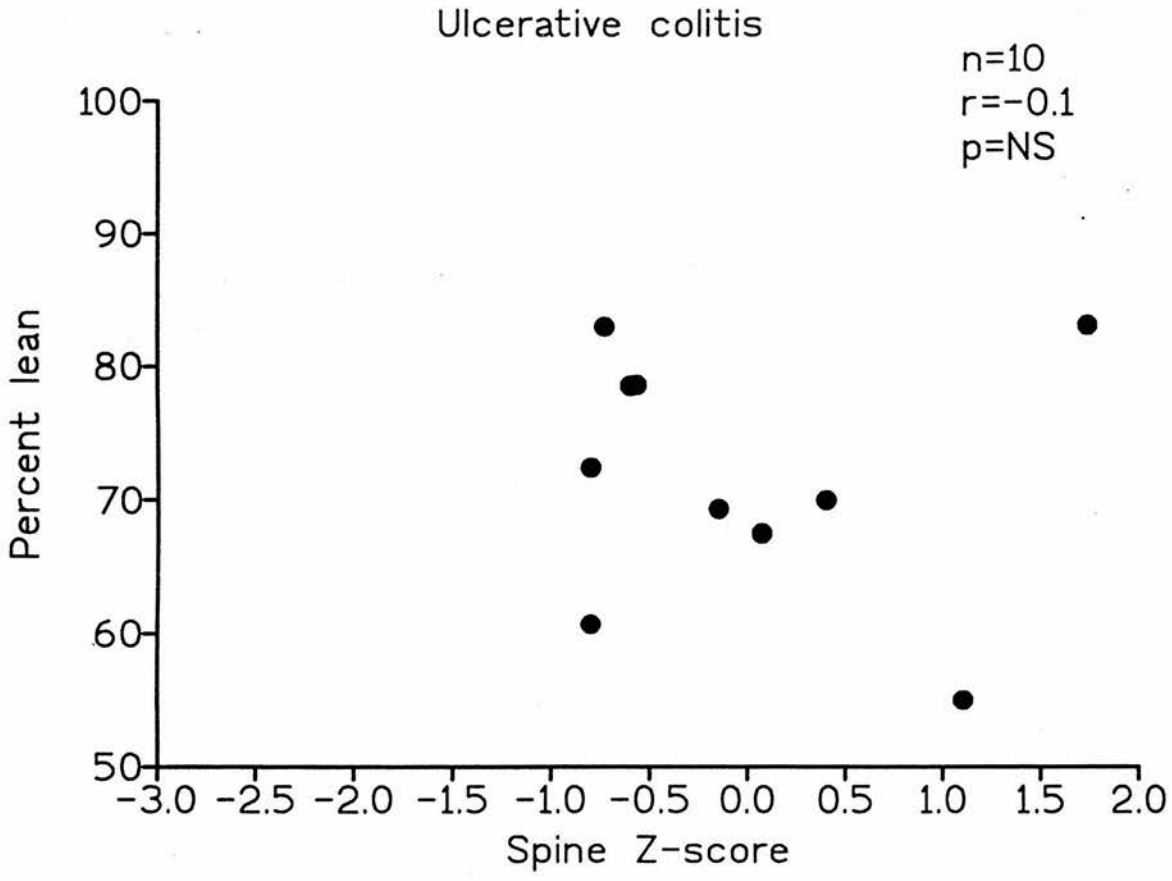

Figure 8.5. Spine Z-scores plotted against percent lean in patients with Ulcerative colitis.

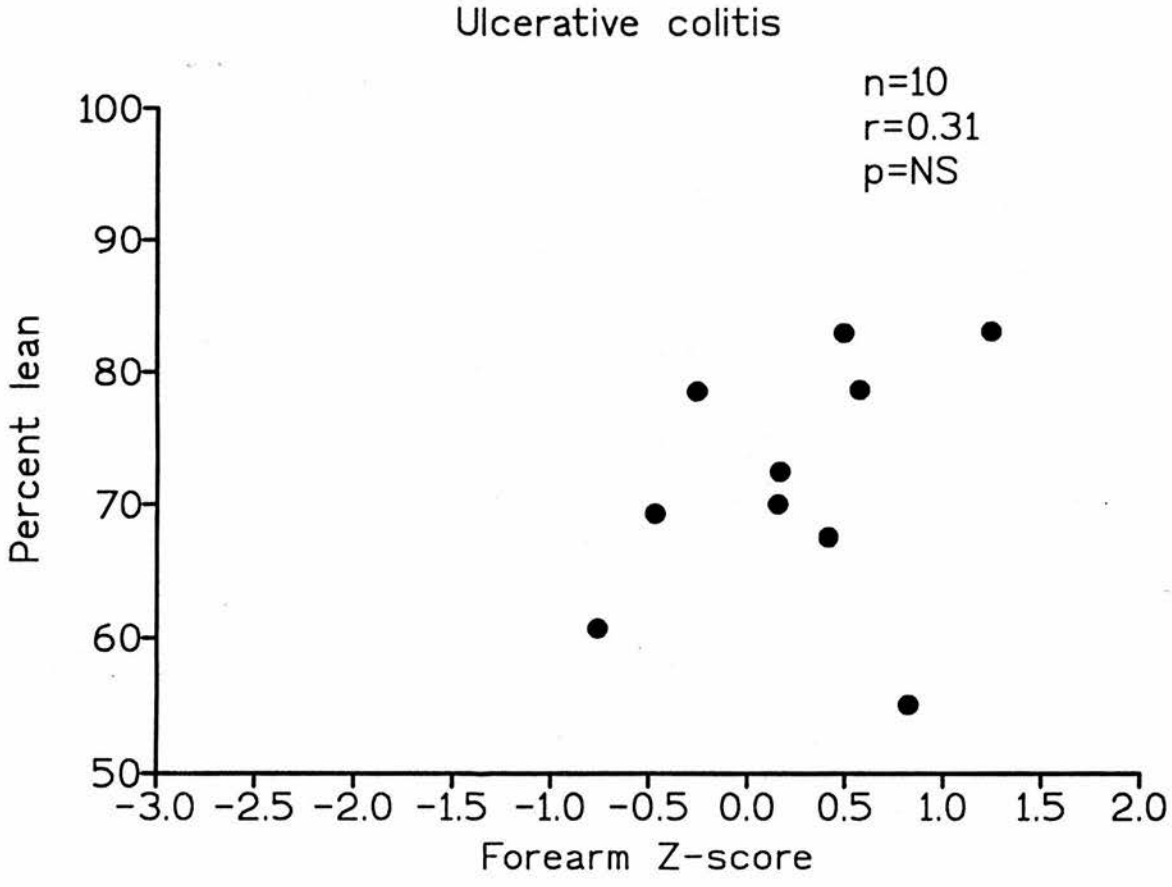

Figure 8.6. Forearm Z-scores plotted against percent lean in patients with Ulcerative colitis.

percent lean, and hence decreased percent fat. There was no correlation of either spine (r=0.09, p=NS) or forearm (r=0.08, p=NS) Z-scores with the BMI.

Figures 8.5 and 8.6 show spine and forearm BMD Z-scores plotted against percent lean in patients with UC. In contrast to CD, there is no correlation of spine (r=-0.1, p=NS) or forearm (r=0.31, p=NS) Z-scores with percent lean.

8.4. Conclusion

In CD, but not in UC, BMD is inversely related to percent lean, i.e., depletion of bone mineral parallels depletion of body fat.

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Chapter IX

BONE MINERAL DENSITY AND GROWTH IN ADOLESCENTS WITH LONGSTANDING INFLAMMATORY BOWEL DISEASE - A SPECIALLY VULNERABLE GROUP

9.1. Introduction

Growth failure and developmental delay are common in children with inflammatory bowel disease (IBD) and should be monitored closely (Kirschner *et al* 1990, Barton *et al* 1990, Mock *et al* 1986). However, data on growth and nutrition in adolescents tend to be from tertiary care centres caring for complicated, difficult disease (Motil *et al* 1993, Hildebrand *et al* 1994, Kirschner 1993) and data from unselected patients from the community are lacking. The Scottish Hospitals' Inpatient Statistics provides a unique epidemiological database which records and links data on all patients admitted to NHS hospitals in Scotland (Kendrick *et al* 1993). Previous work reported serious under-recording of growth and development parameters in a geographically derived cohort of Scottish children with IBD diagnosed during 1968-83 (Barton *et al* 1989a). It is not known whether these parameters have been better monitored recently.

It should be noted that, though there is a large amount of data on growth and nutrition in adolescents with IBD, similar data on bone mineralisation are lacking. Genant *et al* (1976) reported that adolescents were particularly severely affected with osteopenia and growth retardation.

The investigation of adolescents in this thesis involved two separate projects.

1. Investigation of bone mineralisation in adolescents with IBD seen at the Western General Hospital, Edinburgh (i.e. a tertiary referral centre) - to test the hypothesis that they have more severe osteopenia than adults for a given disease duration.
2. An audit of monitoring of growth and development parameters in a geographically defined cohort of Scottish children with IBD and investigation of their morbidity in terms of growth and developmental delay.

9.2. Bone mineral density in adolescents with IBD

I aimed to investigate the effect of IBD on bone mineralisation in adolescents and compare it with that in adults.

9.2.1.Patients:

Spine bone mineral density (BMD) was measured in 5 male and 4 female patients with juvenile -onset Crohn's disease (CD). The median age was 15 years (range 11-19 years). The median duration of disease from the onset of symptoms was 5 years (range 2-13 years). The median duration of corticosteroid use was 17 months. The details of these patients are given in table 9.1. These patients were all complicated, needed prolonged corticosteroid and in 5 patients, immuno-suppressive therapy with azathioprine, and all but one patient (with orogenital CD) had one or more operations, with resectional surgery. Forearm Z-scores were not calculated in these patients as I lacked a suitable age- and sex- matched control population database.

The spine BMD was also measured in 3 adolescents (2 male, 1 female) with ulcerative colitis (UC). They were aged 11, 13 and 16 years. No statistical comparisons were made either with adults with UC or with children with CD, because of small numbers.

Table 9.1. Patient details - adolescents with IBD

CD	
Male:Female	5:4
Median age (range) years	15 (11-19)
Median duration (years)	5 (2-13)
Duration of steroid use (months)	17 (5-64)
Anatomy	Colonic (n=5) Ileo-colonic (n=3) Oro-genital (n=1)
UC	
Male:Female	2:1
Age (years)	11, 13, 16
Disease duration (years)	4, 5, 6
Duration of steroid use (months)	5, 14, 40
Anatomy	pancolitis (n=3)

9.2.2. Methods

Spine BMD was measured by DEXA as mentioned in chapter III. The results were expressed as Z-scores compared with chronological age-sex matched adolescents. Plasma calcium, phosphate (non-fasting), alkaline phosphatase, albumin and 25-OH-vitamin D levels were measured. Radiological bone age was determined in all patients by an experienced radiologist using the Greulich and Pyle reference atlas.

Statistical comparisons were made using the Student's t-test. Correlation of the duration of disease with BMD was made by Pearson's correlation coefficient. The study was approved by the Medicine Subcommittee of the Lothian Area Ethics of Research Committee. Each patient gave informed verbal consent.

9.2.3. Results

The confounding variables that need to be considered in children in interpreting BMD are different from that in the adults. Since calculation of a Z-score depends on a age-sex matched population, delay in growth may affect the apparent Z-score value quite profoundly. Sex hormones have an important role in bone metabolism, and hence a delay in puberty too may affect BMD. The effect of illness on physical activity can be more marked in children than in adults, as their premorbid physical activity is often high.

Two out of the 9 adolescents with CD had clinically severe osteopenia with multiple vertebral collapses (figure 9.1 and 9.2) and back pain needing analgesia. Both were vitamin D deficient and both had particularly complicated, longstanding disease which had needed multiple surgical interventions, prolonged steroid and immunosuppressive therapy and prolonged hospitalisations. At the time of bone density measurements the CDAI scores for these patients were 126 and 92. One of these patients had a bone biopsy at the same time as having a colonic resection; this showed features of osteomalacia in spite of the fact that the patient was on oral vitamin D and calcium supplements.

Figure 9.3 shows the spine Z-scores for bone mineral density in adolescents with longstanding CD. The spine Z-scores for adult patients with longstanding CD discussed in chapter V are also shown for comparison. The mean

spine Z-score in adolescents was -3.1 (SD 1.5) and this was significantly lower ($p<0.01$) than that in the adults (mean -1.1; SD 1.0). It is noteworthy that the two lowest Z-scores in adults were in men with longstanding disease commencing in their teens. There is a significant correlation between duration of disease in years and the spine Z-scores in adolescents with CD ($r = -0.76$; $p<0.02$; figure 9.4).

Figure 9.5 shows the spine Z-scores in the 3 adolescents with UC. The spine Z-scores in adults with longstanding UC discussed in chapter V are also shown. The duration of disease from symptom onset was 5, 6 and 6 years. Though no statistical comparisons were possible in this group, the mean spine Z-score of the 12 adolescents with IBD (-2.9; SD 1.4) was significantly less compared with that in the 27 adult patients with longstanding IBD discussed in chapter V (mean = -1.1; SD 0.8; $p < 0.001$).

The biochemical parameters of calcium metabolism and radiological bone age for each patient are shown in table 9.2.

Figure 9.1. Multiple vertebral collapses in a 18-year-old boy with long-standing complicated Crohn's disease. He was vitamin D deficient and had multiple surgical interventions, total proctocolectomy and received prolonged steroid and immunosuppressive therapy. He had lost 2 cm in height and had severe back pain.

Figure 9.2. Lateral spine radiograph of the boy with Crohn's disease illustrated in figure 9.1 showing multiple vertebral collapses and osteopenia. His spine BMD Z-score was -4.6.

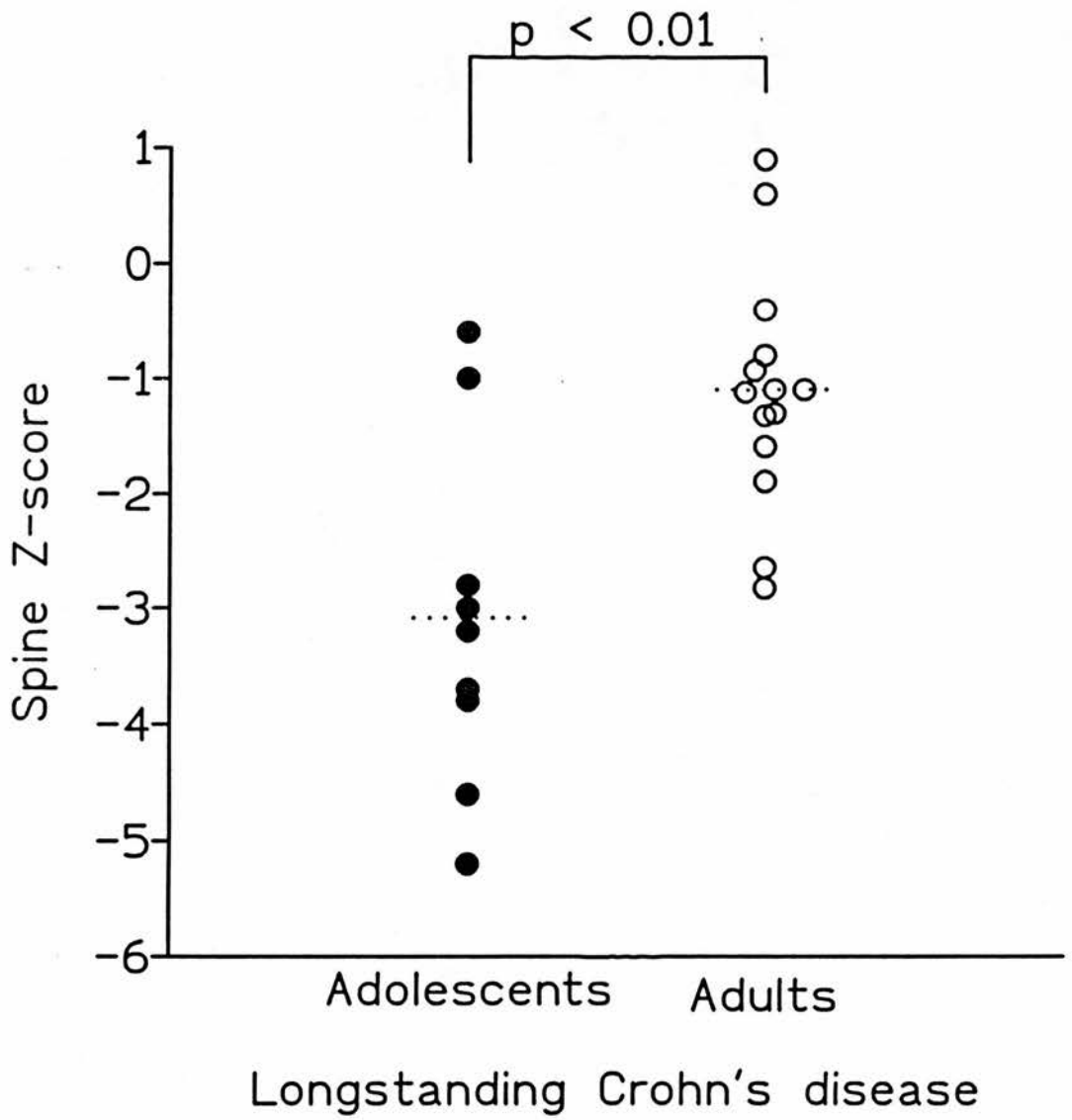


Figure 9.3. Spine BMD Z-scores in adolescents with long-standing Crohn's disease compared with that in adults. The horizontal lines represent mean values.

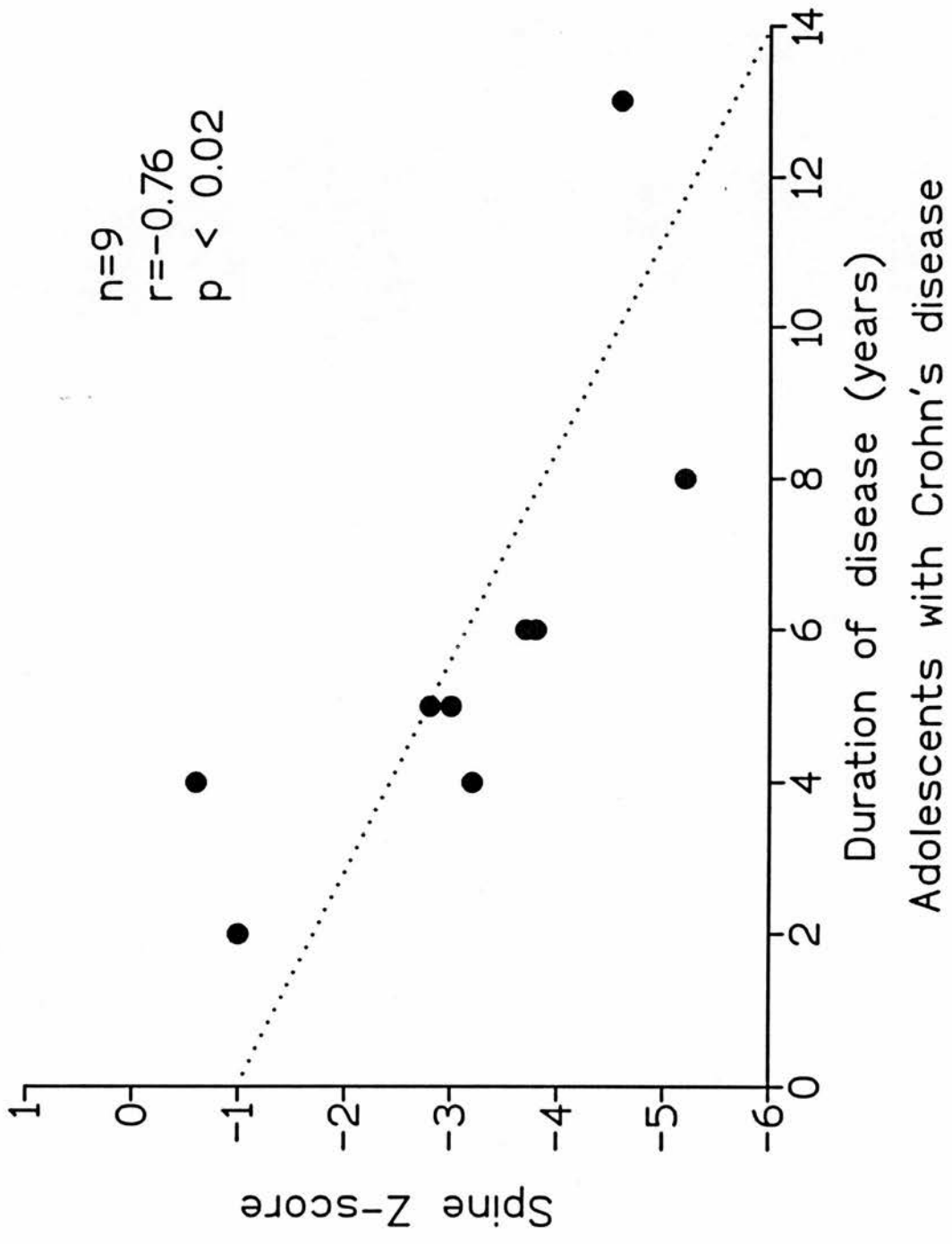


Figure 9.4. Correlation between duration of disease in years and spine BMD Z-score in adolescents with Crohn's disease.

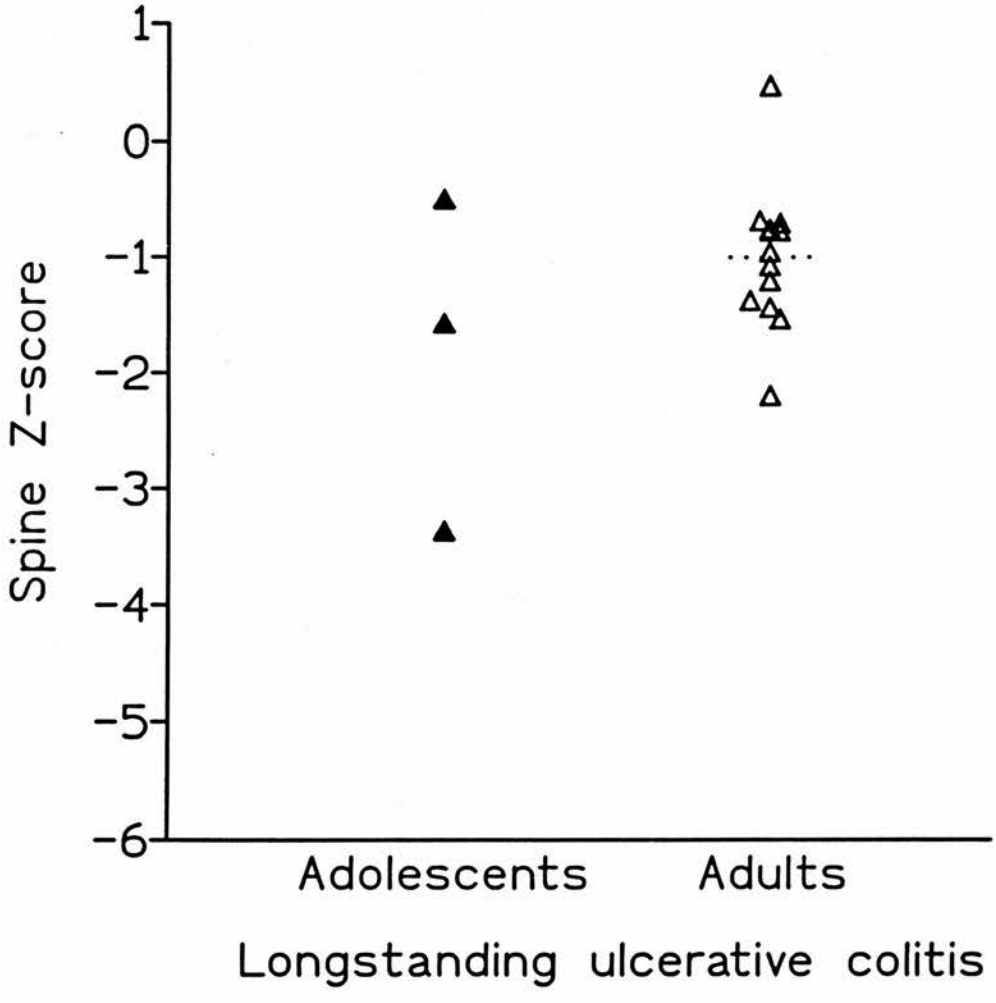


Figure 9.5. Spine BMD Z-scores in adolescents with long-standing ulcerative colitis compared with that in adults.

Table 9.2. Plasma calcium and 25-OH-vit D concentrations and radiological bone ages in adolescents with IBD.

Patient	Chrono-logical age	Sex	Diagnosis	Plasma Ca^{++}(2.1-2.6 mmol/L)Ψ	Plasma 25-OH-vitD (15-100 nmol/L)	Radio-logical bone age (years)
RS*	15	M	CD	2.45	45	13
MM*	11	F	CD	2.26	32	11
LF	13	F	CD	2.34	36	12
DF	16	M	CD	2.20	10	14
ST	19	M	CD	2.36	44	adult
JF	14	M	CD	2.40	48	13
CC	18	M	CD	2.24	8	adult
MD	16	F	CD	2.44	28	16
CS*	13	F	CD	2.32	34	12
FE	13	F	UC	2.42	48	12
PR	16	M	UC	2.30	36	15
CM*#	11	M	UC	2.24	24	11

* pre-pubertal; # primary sclerosing cholangitis; Ψ note change in normal range compared with that in chapter IV.

9.3. Growth and development parameters in juvenile onset IBD

I aimed to determine whether the records of monitoring of growth and development parameters in Scottish NHS hospitals had improved in a more recently diagnosed geographically derived cohort compared with the earlier cohort diagnosed during 1968-83 which was audited by my predecessors. I then investigated the morbidity in terms of growth and developmental delay in this cohort of juvenile onset IBD.

9.3.1. Patients and methods:

Children who had a diagnosis of IBD (UC or CD) and were aged 16 years or less at first admission to a NHS hospital in Scotland were included in this study. The previously reported cohort (Barton *et al* 1990) consisted of 105 children (56 boys and 49 girls), 68 with CD and 37 with UC, who were first admitted between 1968-83. The more recently diagnosed cohort consisted of 41 children (28 boys and 13 girls), 27 with CD and 14 with UC, who were first admitted to NHS hospitals between 1984-88. The inclusion criteria are summarised below:

1. A diagnosis of UC or CD.
2. Age at 1st admission < 17 years.

3. First admission: 1968-83 or 1984-88.
4. Geographically defined cohort:

   1968-83-Lothian, Highland, 10% of rest of Scotland who fulfilled inclusion criteria,

   1984-88-Lothian, Highland, Borders, Fife.

The patients were identified by searching the SHIPS database. Permission was obtained from the consultants caring for the identified patients and case records were obtained from Medical Records department. The case records were scrutinised to confirm the diagnosis and note records of height, weight, radiological bone age and sexual development. Any mention of sexual development was noted though this was often descriptive and not systematically staged. The specialties of the patients' consultants were also noted. A detailed record of date of onset of symptoms, date of diagnosis, surgical interventions, medical therapy, periods of hospitalisations and outpatient visits and all height and weight measurements were entered into a database. The patients were then invited to attend for a structured interview and physical examination. The clinical details of the 1968-83 and 1984-88 cohorts are shown in table 9.3a. Analysis of the 1968-83 cohort was done at a mean of 7 years follow-up, which was the same as the 1984-88 cohort.

The results were analysed using the chi-squared test with Yates' correction.

Table 9.3a. Clinical details of the two cohorts

	1968-83		1984-88	
	CD	UC	CD	UC
n	68	37	27	14
M:F	34:34	22:15	21:6	7:7
Age (yrs) at the time of symptom onset	13(2-16)	13(1-16)	13(4-16)	12(5-16)
Follow-up mean(SD)yr from onset to case-notes scrutiny	7 (4)	7 (5)	7 (2)	7 (3)
Median delay in diagnosis (range) months	4.8 (0-42)	2 (0-61)	3.7 (0-31)	3 (0-10)
Disease anatomy	Small bowel:21 Ileocolonic:26 Colonic:19 Others:2	Pancolitis:17 Left sided:10 Rectum:10	Small bowel:12 Ileocolonic:5 Colonic:10	Pancolitis:8 Left sided:6 Rectum:0

I carried out a structured interview and physical examination of 35 out of the 41 patients in the 1984-88 cohort; the remaining patients had moved addresses and could not be contacted. The clinical details of those interviewed are given in table 9.3b. Height and weight were measured in all patients and radiological bone age was determined in all patients aged 16 years or less and also in patients where a growth delay was suspected. Patients were asked about their parents' heights (or the parents were asked, if present at interview). Mid-parental height centile, an indicator of adult stature, was calculated from father's and mother's height. For boys, his father's height (cm) and his mother's height + 12.5 cm were plotted on a standard nomogram. For girls, her mother's height (cm) and her father's height - 12.5 cm were plotted on a standard nomogram. The height midway between these two points was read from the nomogram and the mid-parental height centile range equals ± 8.5cm of this height.

Table 9.3b. Clinical details of the patients from the 1984-88 cohort who attended the interview and physical examination.

	CD (n=23)	UC (n=12)
Male: female	18:5	5:7
Median age at interview (range)		
male	21 (11-24)yr	21 (17-23)yr
female	17 (16-20)yr	17 (7-23)yr
Disease distribution	Small bowel: n=9 Ileocolonic: n=4 Colonic: n=10	Pancolitis: n=7 Distal colitis: n=5
Active disease at interview (CDAI>150)	6	2
Median follow-up from onset to interview (range)		
male	7 (5-11)yr	9 (6-10)yr
female	6 (5-6)yr	6 (5-7)yr

Non-attenders: CD - 4 patients (3 male, 1 female); similar disease distribution and morbidity as the interviewed group. UC - 2 patients (both male); 1 patient had mild distal colitis, the other had panproctocolectomy and ileostomy.

9.3.2. Results of the audit in the two cohorts

9.3.2.1. Monitoring of growth and development

Height and weight records were considered to be completely unsatisfactory when no records or only one record was available at the time of scrutiny of the case notes. The frequency of growth and development records in the two cohorts is presented in table 9.4. In the 1968-83 cohort, 57 of 105 patients (53%) had completely unsatisfactory height records - it was never recorded in 36 and recorded once in 21 patients. In the 1984-88 cohort, only 6 of 41 patients (15%) had completely unsatisfactory height records (never recorded = 5, recorded once = 1) - a significant improvement ($p<0.001$). The median number of height records in CD disease had significantly improved ($p<0.02$) from 1 (range 0-14) in the 1968-83 cohort to 12 (range 0-37) in the 1984-88 cohort. The corresponding data for UC was 1 (range 0-18) and 17 (0-56) ($p<0.02$). Weight records were completely unsatisfactory in 13 of the 105 (12%) in the 1968-83 cohort (never recorded = 9, recorded once = 4); however, all patients in the 1984-88 cohort had weight recorded at least thrice ($p<0.05$). The median number of weight records in CD and UC was 5 (range 0-15) and 5 (0-42) in the 1968-83 cohort and 23 (range 3-41) and 22 (range 3-56) in the 1984-88 cohort ($p<0.05$).

Radiological bone age was determined in 19 out of 105 patients (18%) in the 1968-83 cohort, but in 17 out of 41 (41%) in the 1984-88 cohort ($p<0.01$). Sexual development was recorded in 29 of the 105 patients (28%) in the 1968-83 cohort and in 22 of the 38 (3 children were excluded as they were still prepubertal at review) patients (58%) in the 1984-88 cohort ($p<0.01$). However, mention of sexual development was frequently only descriptive and not systematically staged.

In both the 1968-83 and 1984-88 cohort, documentation of growth and development parameters were similar for rural and urban regions and for teaching and district hospitals. In the 1984-88 cohort, a total of 407 outpatient clinic visits for the 27 patients with CD were scrutinised - each patient was seen a median of 55% of visits by a consultant (range 0-100%). The 14 UC patients needed a total of 282 outpatient clinic visits - each patient was seen a median of 66% of visits by a consultant (range 38%-94%).

Table 9.4. Comparison of growth and development records in the two cohorts of children with inflammatory bowel disease.

Records	1968-83		1984-88	
	CD(68)	UC(37)	CD(27)	UC(14)
Height:				
Never recorded	20(29%)	16(43%)	4**(15%)	1**(7%)
Recorded once	16(24%)	5(14%)	0	1*(7%)
Median no ht records/patient (range)	5(0-15)	5(0-42)	23**(3-41)	22**(3-56)
Weight:				
Never recorded	4(6%)	5(14%)	0	0
Recorded once	1(1%)	3(8%)	0	0
Median no wt records/patient (range)	5(0-15)	5(0-42)	23*(3-41)	22*(3-56)
Any bone age record	15(22%)	4(11%)	13*(48%)	4(29%)
Any sexual development record	24(35%)	5(14%)	17*(65%)	5(42%)

*p<0.05; **p<0.02

9.3.2.2. Growth parameters in the two cohorts at diagnosis

Table 9.5 shows the frequency of height retardation at diagnosis in the two cohorts, where this data had been recorded in the case notes. There was no statistically significant difference between the two cohorts, either for male or for female patients. Height retardation was a feature of CD but not of UC. In the two cohorts, 14 out of the 61 patients with CD were below the 3rd centile in height, whereas only 1 out of 26 patients with UC was below the 3rd centile in height ($p<0.05$).

Table 9.5. Frequency of low height at diagnosis:

	1968-83		1984-88	
	CD (n=68)	UC (n=37)	CD (n=27)	UC (n=14)
< 3rd centile				
Male	6/19 (32%)	0/10	3/16 (19%)	0/4
Female	5/21 (24%)	1/5 (20%)	0/5	0/7
3-50th centile				
Male	4/19 (21%)	7/10 (70%)	11/16 (69%)	2/4 (50%)
Female	16/21 (76%)	2/5 (40%)	4/5 (80%)	4/7 (57%)

Table 9.6 shows the frequency of low weight at diagnosis in the two cohorts. Again there was no significant difference in frequency between the two cohorts. Fortyfour out of the 81 patients in the two cohorts with CD where records were available were below the 3rd centile in weight, while only 1 out of 41 patients with UC was below 3rd centile in weight ($p<0.001$). Females with CD were underweight significantly more frequently than males ($p<0.01$). Hence, height and weight retardation at diagnosis is a feature of CD but not UC. It may be noted from table 9.3a that the delay in diagnosis (symptom onset to diagnosis) had not changed in the more recently diagnosed cohort.

Table 9.6. Frequency of low weight at diagnosis

	1968-83		1984-88	
	CD (n=68)	UC (n=37)	CD (n=27)	UC (n=14)
<3rd centile				
Male	11/28 (39%)	0/17	8/19 (42%)	0/5
Female	21/28 (75%)	0/12	4/6 (67%)	1/7 (14%)

It is interesting that there was no significant improvement in the frequency of height and weight retardation at diagnosis in the 1984-88 cohort compared to the 1968-83 cohort, because there were other aspects of morbidity which showed evidence of improvement in the 1984-88 cohort compared with the 1968-83 cohort. These are shown in tables 9.7- 9.10. There was a significant reduction in the number of patients with CD having resectional surgery or laparotomy in the more recently diagnosed cohort (table 9.7). There was also a significant reduction in the inpatient hospital stay for patients with CD in the more recently diagnosed cohort (table 9.8), though the number of admissions were unchanged. All deaths occurred prior to 1978. The results would indicate that though there was no change in morbidity in growth and development at diagnosis, better medical management in the more recently diagnosed cohort led to shorter hospital stay and fewer surgical resections in CD. It may be seen from table 9.10 that juvenile onset CD patients were more frequently treated with systemic steroids, azathioprine and enteral nutrition with elemental diet in the 1984-88 cohort compared with the 1968-83 cohort.

Table 9.7. Surgery

	1968-83		1984-88	
	CD (n=68)	UC (n=37)	CD (n=27)	UC (n=14)
No. having major surgery	49 (72%)	11 (30%)	13* (48%)	3 (21%)
No. having gut resection	39 (57%)	8 (22%)	11** (41%)	3 (21%)
No. with perm. stoma	10 (15%)	8 (22%)	1 (4%)	1 (7%)
Ileoanal pouch	0	0	0	2 (14%)

*p<0.05; **p<0.01

Table 9.8. Admissions & Inpatient days

	1968-83		1984-88	
	CD (n=68)	UC (n=37)	CD (n=27)	UC (n=14)
No. of admissions. Median (range)	4 (1-13)	3 (1-20)	5 (1-15)	4 (1-16)
Inpatient days. Median (range)	64 (7-332)	30 (1-273)	36* (8-415)	32 (8-115)

p<0.05

Table 9.9. Deaths

	1968-83		1984-88	
	CD	UC	CD	UC
No of deaths	5*	1*	0	0

*All deaths occurred in the early part of the study period before 1978.

Table 9.10. Medical therapy

Treatment	1968-83		1984-88	
	CD (n=68)	UC (n=37)	CD (n=27)	UC (n=14)
Steroids				
Systemic	48(71%)	21(57%)	25(93%)*	12(86%)
Local	15(22%)	22(59%)	6(22%)	12(86%)
none	5(7%)	6(16%)	2(7%)	1(7%)
SZP	49(72%)	33(89%)	19(70%)	11(79%)
Other 5ASA	0	0	12(44%)	3(21%)
Azathioprine	10(15%)	2(5%)	12(44%)**	3(21%)
Metronidazole	7(10%)	1(3%)	5(19%)	0
Cyclosporin	0	0	1(37%)	0
Enteral nutrition	11(16%)	1(3%)	12(44%)**	1(7%)

*p<0.05; **p<0.02

9.3.2.3. Growth parameters at the time of interview

Height and weight were measured in the 35 out of the 41 patients who attended for interview. The heights of the patients were indexed against the calculated mid-parental height centile, an indicator of adult stature obtained from a standard nomogram. Mid-parental height centiles could be calculated in 33 out of the 35 patients. The results are shown in table 9.11.

Table 9.11. Patients' height compared with the mid-parental height centile range

	Crohn's disease	Ulcerative colitis
No. below mid-parental centile range		
male	5*/16 (31%)	0/5
female	0/5	2♠/7 (29%)
No within mid-parental centile range		
male	11/16	5/5
female	5**/5	5♣/7

* three patients less than 16 years old and one 17 years old but still growing.

** two patients still growing.

♠ aged 7 and 10 years.

♣ one patient still growing.

Table 9.12 shows the percent ideal body weight of the 1984-88 cohort of juvenile-onset IBD patients at the time of interview. Six patients with CD and two with UC had active disease at interview (table 9.3b) and the percent ideal body weights of these patients are compared with those with inactive disease in figure 9.6. The median percent ideal body weight of the patients with active disease (n=8; 79%; range 59-113%) was significantly lower ($p<0.01$; Mann-Whitney U- test) than that in those with inactive disease (n=26; 95%; 80-159%).

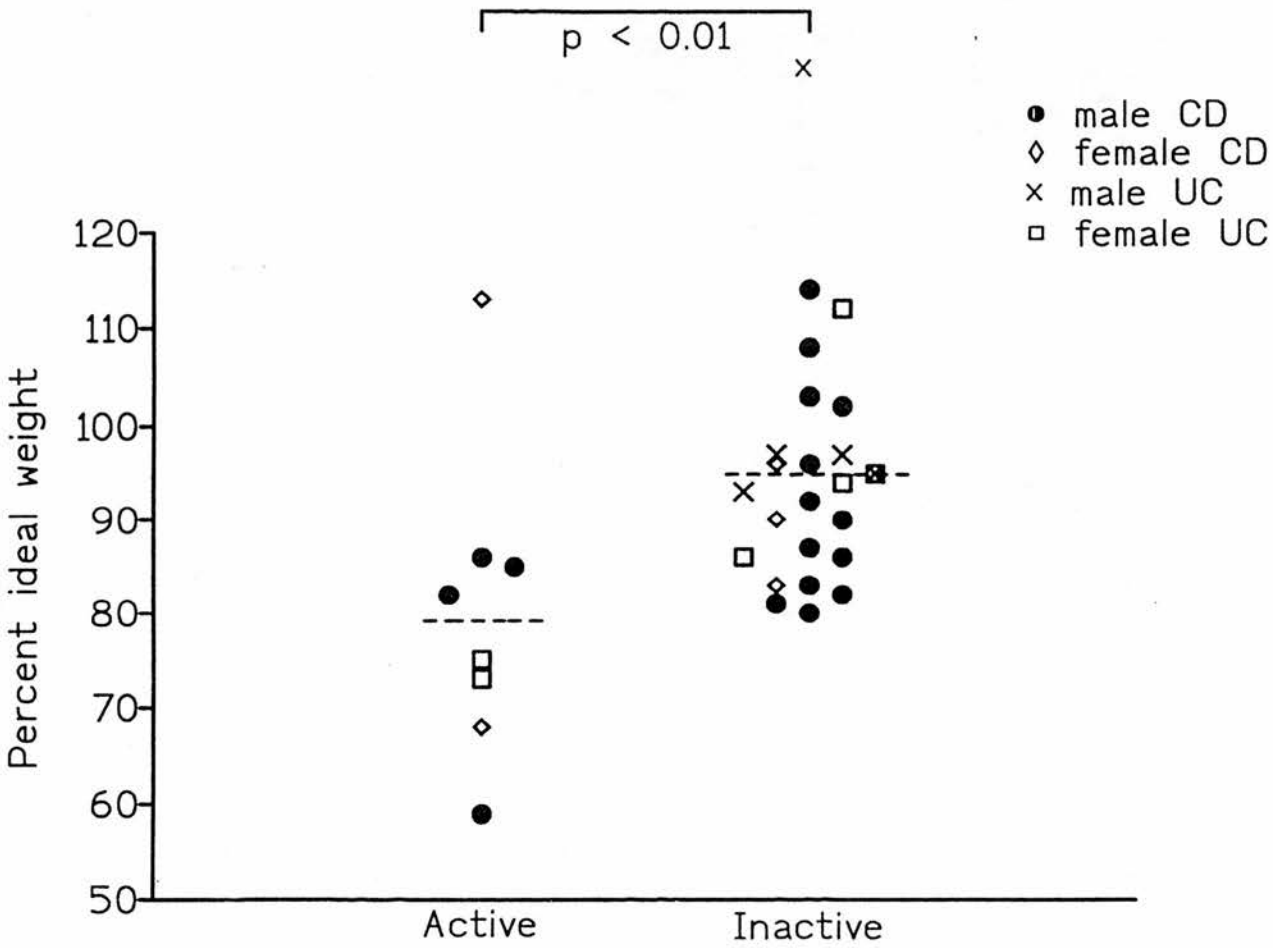


Figure 9.6. Comparison of the percent ideal body weights of those with active juvenile-onset IBD with those with inactive disease.

Table 9.12. Percent ideal body weight (median & range) of the patients interviewed. One female patient with ulcerative colitis was pregnant at the time of interview and her data is not included.

	Crohn's disease	ulcerative colitis
Male (n=23)	86 (59 - 114)	97 (93 - 159)
Female (n=11)	90 (70 - 116)	90 (73 - 112)

Table 9.13 shows the radiological bone ages and chronological bone ages in the 6 patients less than 16 years old at interview, and in the 3 patients older than 16 years where growth delay was suspected. Four out of the 6 patients with CD and one out of 3 patients with ulcerative colitis had growth delay.

Table 9.13. Radiological bone ages at interview in the 1984-88 cohort.

Diagnosis	Sex	Chronological age (years)	Radiological bone age (years)
CD	M	14.7	14
CD	M	11.3	11
CD	F	15.6	13
UC	F	7	7
UC	F	10	10
UC	F	14.9	13
CD	M	16.1	12
CD	M	17.3	16
CD	M	17.2	14

9.4. Conclusions

Adolescents with chronic IBD referred to a tertiary centre are more severely osteopenic than adults with a similar duration of disease at the same tertiary centre. The growing bones probably suffer the brunt of the osteopenic effects of disease and treatment. Serious vertebral collapse and loss of height may occur. The bone mineral density is inversely correlated with duration of disease.

Significant improvements in recording growth and development parameters had taken place in the 1984-88 cohort compared to the 1968-83 cohort. Our previous report of the deficiencies in recording these parameters was published in 1989 and hence the improvement in the 1984-88 cohort cannot be ascribed to the greater awareness resulting from this publication - the frequency of recording these parameters was not greater after 1989 compared to that prior to 1989 for the 1984-

88 cohort. Deficiencies were however still present in recording height, sexual development and bone age. Greater awareness of this aspect of morbidity of inflammatory bowel disease in children is needed.

Height and weight retardation at diagnosis were features of juvenile onset CD but not UC. The frequency of height and weight retardation at diagnosis had not changed in the more recently diagnosed cohort, though other aspects of morbidity, such as the frequency of surgical resections and inpatient stay in hospital had improved, probably as a result of better medical management.

Both studies show the profound nutritional effects of CD in children and adolescents.

♦♦♦

Chapter X

DISCUSSION : BONE MINERAL DENSITY AND BODY COMPOSITION IN INFLAMMATORY BOWEL DISEASE

In this chapter I have discussed the results presented in chapters IV - IX. Discussion of bone mineral density (BMD) in IBD is followed by discussion of body composition in adults and analysis of data specifically relevant to children.

10.1. Bone mineral density in newly diagnosed IBD

I have not only confirmed many other reports of low bone mineralisation in IBD but have clearly shown that at the time of diagnosis this is a feature of Crohn's disease (CD) but not ulcerative colitis (UC). The two groups of patients were well matched with regard to age, sex, severity and estimated duration of disease prior to diagnosis. The lower BMD in patients with CD applies equally to trabecular bone comprising 50% of the lumbar spine (Newton *et al* 1993) and to cortical bone found in the forearm, and the degree of bone demineralisation is not correlated with CDAI or the BMI. Though physical activity was reduced at the time of diagnosis compared with usual activity levels, the grades of activity were similar for UC and CD patients. Malabsorption is unlikely to be a major factor in the aetiology of bone loss as patients with colonic disease had bone densities similar to those with small bowel involvement. This is further supported by the fact that bone metabolic parameters were normal and there were no differences between patient groups. However, calcium or vitamin D deficiency may exist in spite of normal biochemistry and bone histology would be needed to definitely exclude this possibility.

In view of the small numbers involved, the lack of difference in BMD Z-scores between small bowel and colonic CD, and between smokers and nonsmokers must be interpreted with caution as type 2 errors cannot be excluded. Further studies with larger number of patients are needed before firm conclusions may be reached. Only 1 UC patient was an ex-smoker. She had stopped smoking 3 years prior to presentation after smoking for 5 years. Her spine Z-score was -0.5 and forearm Z-score 0.1. Excluding her from the nonsmoker group did not alter the statistical lack of significance between smokers and nonsmokers.

The results of this study underline the fact that CD and UC are different diseases. CD is a systemic disease with a long pre-morbid phase, while UC is a mucosal disease with an acute onset. It had been shown previously in a study of Scottish children with IBD that 11 out of 40 young children with CD were below the third centile for height while none out of the 14 children with UC was below the third centile for height (Barton *et al* 1990).

Maintenance of BMD should not be equated with the important issue of linear growth in growing children. The effect of IBD and its treatment on growth in children has been studied by various workers including my predecessor (Barton *et al* 1990). The present study is predominantly on an adult population and extrapolation to growing children should be done with caution. Interpretation of BMD in adolescents need additional considerations. Adolescents going through puberty at different ages may be an important confounding factor in interpreting serial BMD measurements, particularly if the patients are going through puberty at different ages from the control adolescents. In this study, however, only 4 patients (2 CD, 2 UC) were going through puberty and only 1 patient with CD had delayed puberty. All the remaining patients were sexually mature adults. The patient with CD and delayed puberty was a 14-year-old boy with spine Z-score of -1.5 at diagnosis and -1.8 after 1 year. Delay in growth may be a confounding variable if comparison is made with adolescents with normal growth. In this study only the 14-year-old boy referred to above had a bone age less than chronological age with height on the 3rd centile and recalculation of his Z-score using bone age did not alter the significance level of the difference between CD and UC in spine Z-scores. Since forearm Z-scores were only available in adults aged 20 or over, these results do not suffer from the confounding influence of growth and sexual development. Despite the fact that 20 of 23 patients received systemic corticosteroid therapy, at 1-yr follow-up there was no evidence of any further bone loss either in CD or in UC. As a result of treatment, only 2 patients overall (1 CD, 1 UC) had continuing active disease after 1 year - other series who report continuing loss of bone during follow up have not been so successful in treating their patients.

Several studies have confirmed that osteopenia in patients with IBD is unassociated with calcium homeostasis defects (Compston *et al* 1987, Stallmach *et al* 1988, Motley *et al* 1988, Bernstein *et al* 1993, Bjarnason *et al* 1993). Two recent

studies reported as abstracts have recorded greater prevalence of osteopenia in CD compared with UC (Tromm *et al* 1993, Bernstein *et al* 1993). The two studies however identify ileal involvement and greater steroid use respectively as the factors responsible for the difference from UC. None of these factors appeared to be relevant in this study.

The study failed to confirm the rapid bone loss reported previously (Motley *et al* 1988) in some patients with IBD. In my series of patients, therapy generally resulted in remission of activity of the disease with no further bone loss; indeed, a slight increase in Z-scores occurred in some patients. Studies that have randomly recruited patients from follow-up outpatient clinic for bone density studies are likely to be biased towards patients with complicated long-standing disease as they are likely to attend more frequently. However, I reiterate that my follow-up period is only 1 year and the conclusions should not be extrapolated to patients on more prolonged steroid therapy.

The mechanism of osteopenia in CD is unclear. The possibility that there is a primary disorder of osteogenesis so that bone formation is less than normal cannot be excluded. Abnormality of the mononuclear phagocytic system resulting in excessive resorption by osteoclasts or mediators from the inflamed gut triggering osteoclast activity are other possibilities which need investigation. Bone resorption is mediated by the unique multinucleated bone cell, the osteoclast, the formation and activity of which is regulated by a family of cytokines (Raisz *et al* 1988). The cytokines that regulate osteoclast function include IL-1, IL-6, TNF-α, TGF-α, γ interferon, IL-1 receptor antagonist and IL-4 (MacDonald *et al* 1992). Various cytokine abnormalities have been described in CD (Mazlam *et al* 1992, Fais *et al* 1991) and differences from UC have been highlighted. Further studies are needed to elucidate the interaction of osteoclasts and cytokines in CD.

The findings in this study may have important clinical implications. It is likely that control of disease activity with appropriate therapy may actually improve bone mineralisation which may counterbalance the known osteopenic effects of steroid therapy. Effects of other interventions such as regular exercise, calcium and vitamin D supplementation to offset gut losses and hormone replacement therapy in postmenopausal women need to be examined. Though small numbers make it impossible to analyse the effects of secondary amenorrhoea and hormone replacement therapy, the results show that it is not a confounding factor in

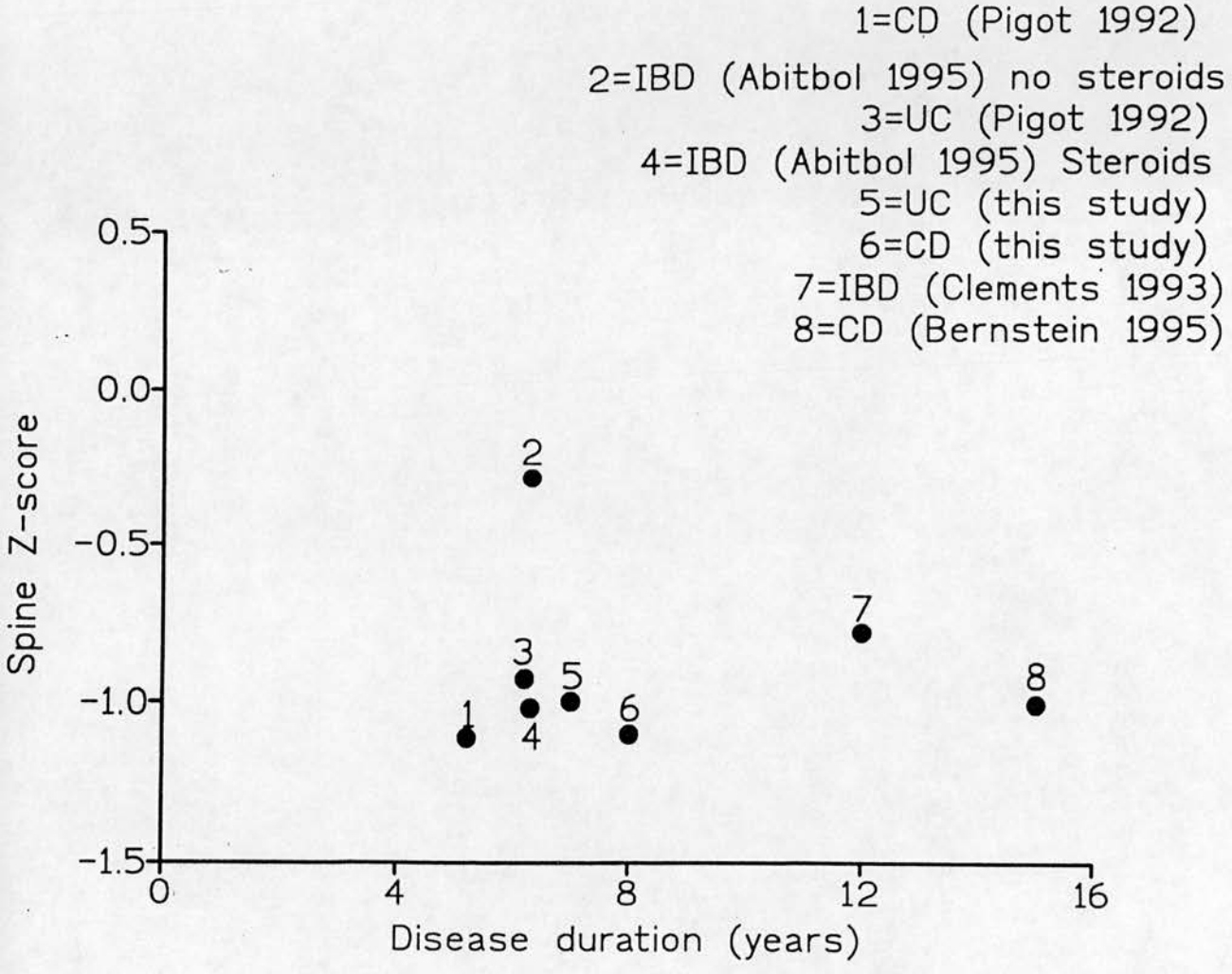
explaining the difference between CD and UC. A recent study has reported prevention of bone loss in postmenopausal women by hormone replacement therapy (Clements *et al* 1993). In order to assess results of interventions it is important to identify cases with intrinsic low bone mineralisation and those at risk of accelerated bone loss.

10.2. Bone mineral density in longstanding IBD

It was important to explain why other published studies did not find any difference in BMD between CD and UC. The results in patients with long-standing IBD provide an explanation. Previous studies (Clements *et al* 1992, Compston *et al* 1987, Pigot *et* al 1992, Bernstein *et al* 1995) had recruited unselected patients from those attending a tertiary referral centre. Such cohorts may be biased towards long-standing complicated disease and indeed, the average duration of disease in the published series ranged from 5 to 10 years. I deliberately recruited a cohort with long-standing disease - median disease duration of 8 years for CD and 7 years for UC. All patients had inactive disease to further avoid the direct effect of disease as shown in my study on newly diagnosed patients.

The results show that there was no difference in mean BMD between CD and UC. The two groups of patients were well matched in the confounding variables, such as duration and cumulative dose of steroids and in the grade of physical activity. In CD, it was interesting to note that the mean Z-score in the patients with long-standing disease was almost identical to that in the newly diagnosed cohort at diagnosis and after one year of follow up. The mean Z-scores reported by other groups were also very similar. This is illustrated in figure 10.1. The usual biochemical parameters of calcium homeostasis were normal and this was the experience of all previously published studies which had measured these parameters (Compston *et al* 1987, Pigot *et al* 1992, Motley *et al* 1988, Abitbol *et al* 1995).

In UC, disease duration and spine and forearm Z-scores were significantly correlated inversely. A similar inverse correlation could be demonstrated between cumulative steroid dose and BMD Z-scores. In contrast, in CD disease, no such correlation could be found between BMD and duration of disease or with cumulative steroid dose. This would indicate that duration of disease and cumulative steroid dose are important determinants of osteopenia in UC. In CD, the



10.1. Average duration of disease (mean or median) and mean spine Z-scores in IBD reported by different authors, including my study, on long-standing IBD (chapter V). The mean spine Z-scores were not related to disease duration, but all studied patients with long-standing disease.

situation is more complicated - osteopenia caused by the disease itself is counterbalanced by effective treatment with steroids which itself induces some bone loss.

10.3. Mechanism of osteoporosis in patients with IBD

Results of bone turnover markers were available only from a small number of patients with newly diagnosed IBD prior to treatment and I am cautious in drawing any firm conclusions. Urinary pyridinium crosslinks excretion, a marker of bone resorption was increased in some patients with CD, but in none with UC. Plasma osteocalcin and bone alkaline phosphatase, bone formation markers, were close to normal levels in all patients. The results contradict the finding of low serum osteocalcin concentration in IBD reported by Abitbol *et al* 1995. It must be noted that more than half of the patients studied by this French group had received steroid therapy which decreases serum osteocalcin level (Reid *et al* 1986). In contrast, Bjarnason *et al* 1994 showed normal osteoblast markers and increased osteoclast markers in IBD. Increased bone resorption in some newly diagnosed CD patients prior to institution of therapy found by me would support the hypothesis of cytokine-induced activation of osteoclasts, as detected *in vitro* (Bertolini *et al* 1986). However, as discussed later in chapter XVI, gut production of pro-inflammatory cytokines such as IL-1β and IL-6 studied by the technique of whole gut lavage fluid analysis showed no difference between CD and UC.

In this study, it could not be established that the changes in BMD found in this study are related to systemic inflammation. No correlation was found between CDAI and BMD Z-scores and other indices of systemic inflammation such as CRP, platelet count and white cell count did not correlate with BMD Z-scores. Serum IL-6 was not measured in this study.

10.4. Statistical considerations

The number of IBD patients in the BMD studies mentioned above are small, and the findings are prone to type II error. However, combining the newly diagnosed patients in chapter IV and chapter VI gives 21 CD and 19 UC patients. This gives 80% power to detect a significant difference in Z-scores at p=0.05 level. In chapter V, the number of patients with long-standing IBD is too small to exclude

a type II error with confidence. However, further experience with much larger number of patients with long-standing IBD (manuscript submitted for submission) supports the lack of difference between CD and UC. As a consequence of the small numbers, multiple discriminant analysis of the relevant factors affecting BMD (such as smoking, menstrual status, oral contraceptive pills) did not provide meaningful results. Data on these factors are however provided to establish the fact that CD and UC patients were well matched as to these confounding variables.

10.5. Methods of assessing body composition

The ideal instrument for assessment of body composition in patients should yield accurate and reproducible results, be inexpensive, portable, easy to operate and to interpret the readings, safe for the patient, should not involve irradiation, and repeated measurements should be possible. None of the available methods fulfill all, or even most, of these criteria and clearly this explains the limited use of body composition data in clinical practice. Furthermore, there are no accurate reference data for the general population on the lean:fat ratio, analogous to the regularly updated information which is collected on height, weight and BMI, related to age, sex, UK region and social class.

The lightweight, portable *hand-held* bioelectrical impedance analyser used in the present study proved to be a very satisfactory instrument for measuring body composition. The evaluation against two standard instruments currently used for such studies by medical physicists showed that the three gave virtually identical values for lean body mass in normally nourished and undernourished adults. The Bland and Altman method showed satisfactory agreement in LBM measurements between DEXA and the *hand held* machine with a bias of 2.6%. However, the limits of agreement can be -9% to 14%. Since the *hand-held* and *in-house* machines gave identical readings of bioelectrical impedance, the portable instrument should also be satisfactory for the assessment of lean and fat in children, once prediction equations appropriate to this age group have been developed.

Non-medical staff could be taught to use the machine in a few minutes, the program is driven by a simple menu and the results could be directly read out and stored without the need for additional software or access to a computer. If further

studies in larger clinical groups support our current findings, this type of instrument is likely to have wide applications in medical practice and could also be used to collect reference population data. Since the patient has to be weighed in order to calculate lean body mass, some degree of mobility is necessary.

Apart from macerating a corpse (which has been done!), every technique for measuring body composition has theoretical problems and will be unsuitable for certain groups of patients. In studies of obesity, quite different criteria are used to select an appropriate method, than will be used for research in starvation, malabsorption or IBD. Studies in my institution which involved comparisons with prompt neutron activation analysis (PNAA) and total body water (TBW) determination using tritiated water have shown that DEXA is a reproducible and accurate method of estimating lean body mass (Hannan *et al* 1994). I now report that lean body mass values, derived from impedance measurements made with a hand-held instrument (costing £400) were almost identical to that measured by DEXA (using a machine which cost £80,000 six years ago), both in healthy and diseased subjects. Use of prediction equations derived for specific disease groups (i.e. eating disorder, IBD) instead of the manufacturer's built-in equation for the hand-held machine, actually worsened the correlation with values obtained by DEXA.

The limitations of BMI in respect of the proportions of lean and fat are well recorded (Garn *et al* 1986, McLaren 1987, Smalley *et al* 1990), but this is not widely appreciated by clinicians. Though decrease or increase in both lean and fat occur with weight loss or weight gain, the relationship between the two compartments may change. A good example of this is the increase in percentage of body mass as lean, associated with the use of anabolic steroids. The data illustrate that undernourished patients segregate into different groups predominantly depleted either of fat, of lean, or of both. Clearly, both underlying pathogenesis and appropriate management of the undernutrition, will differ greatly in these groups. More widespread use of two-compartment body composition analyses as well as body mass index in these situations should provide valuable information on the complex interplay between low nutrient intake, inflammation, nutrient loss and the effects of physical inactivity and of drugs. Body composition analysis will also ensure accurate and universal recording of height and weight,

basic nutritional parameters often neglected in clinical practice (Barton *et al* 1989a).

The two compartment model, although invaluable conceptually, is still a highly over-simplified statement of the clinically relevant body constituents in states of undernutrition. The "fat" compartment includes brain, and the non-fat "lean" mass includes total body water, visceral proteins and bone mass. In stable conditions, total body water is remarkably constant at 73.2% of lean (Lukaski 1987), and this principle underlies the use of total body water by isotope dilution techniques, in studies of body composition. However this also means that data on so-called "lean" will be misleading in the presence of oedema, dehydration, ascites, cardiac failure, and in conditions with rapid fluid shifts such as the immediate postoperative period. In patients on TPN, some shift in body water may be anticipated.

I have presented our findings as percent body weight as lean, and discussed the data in terms of percent fat or percent lean. However, with further experience and larger numbers of patients studied, and particularly when reference population data are available, better and even more informative ways of expressing these data may emerge.

The primary purpose in this work was to improve our clinical assessment of patients with gastro-intestinal diseases, particularly IBD. Young adults with CD are abnormally thin (Ferguson *et al* 1994b), and newly diagnosed patients with CD have significant bone demineralisation (Ghosh *et al* 1994). These features are not associated with a particular distribution of disease or inflammatory disease activity, nor with corticosteroid treatment. Results from the few IBD patients in the present study further emphasise the heterogeneity of nutritional status in these diseases. A simple technique for assessing body composition will further improve the capacity to analyse the many contributions to overall illness in IBD and other diarrhoeal diseases as a basis for rational treatment (Ferguson *et al* 1994a).

10.6. Body composition in IBD

In chapter VIII, I have presented body composition analysis data in IBD patients. It again emphasises the nutritional morbidity of CD disease compared with UC. Ten patients had a BMI less than 20 and nine of them had CD (Figures 8.1

and 8.2). The only patient with UC who was underweight was a young girl with a definite history of anorexia nervosa needing psychiatric support. The data support the contention made in the previous section that in IBD, underweight patients often have lost proportionally more fat than lean, as both for male and female patients, a significant inverse correlation could be observed between the percent lean and BMI. An interesting observation was the inverse correlation between spine and forearm Z-scores and percent lean in CD but not in UC. Bone is part of the lean compartment, and one would normally expect a reduction in bone mass to be accompanied by a reduction in percent lean; in CD, osteopenia parallels depletion of body fat. In chapter II I have discussed the theoretical basis for the hypothesis that metabolic alterations resulting from inflammation play a role in nutritional abnormalities in systemic inflammatory conditions such as CD. Both bone and fat metabolism are influenced by classical 'cachectins' TNF-α and IL-1β (Raisz 1988, Bertolini *et al* 1986, Camussi *et al* 1991, Grunfeld *et al* 1992), as discussed in chapter II. Though indirect, the evidence in this thesis suggests that further experimental work in this area is warranted. Evidence implicating TNF in causing malnutrition in chronic IBD is, however, lacking (O'Connell *et al* 1994).

10.7. Special considerations in adolescents

Adolescents with IBD are worthy of special consideration. Studies in our institution (Barton *et al* 1990, Ghosh *et al* 1994c) and in other centres (Griffiths *et al* 1993, Hildebrand *et al* 1994, Motil *et al* 1993) have reported considerable nutritional morbidity in this group of children, especially in those with CD. Though the cohort of adolescents recruited for the bone mineralisation study consisted of complicated patients referred to a tertiary care centre, the degree of osteopenia present in them was certainly worrying. In some but not in all cases this was accompanied by vitamin D deficiency. Osteopenia was also present in adolescents with long-standing UC who had received considerable cumulative steroid doses. Vertebral collapse and loss in height were seen in two of the patients, testifying to the potential devastating effect of osteopenia in this age group. Growing bones are metabolically very active and it is possible that they are susceptible to multiple metabolic influences very easily. Lack of physical activity almost certainly is another contributing factor, though in my study, it was impossible to obtain an accurate measure of their premorbid physical activity status as the onset of illness

was several years prior to measurement of BMD. Issenman *et al* 1993 reported low bone density in paediatric CD compared with normal children, though the difference was less when compared with height-matched controls. Again, adequate treatment with steroids (alternate-day) over 2 years did not result in further bone loss. Growth and sexual development are obviously important factors related to bone density in children (Issenman *et al* 1993) and both are directly affected by CD in childhood (Barton *et al* 1990).

It was important to investigate the nutritional morbidity in unselected patients from the community, rather than the selected group discussed above, and the audit of delivery of medical care to Scottish children provided an ideal opportunity to look at a geographically defined cohort. It is fortunate that Scotland has an excellent epidemiological tool in the Scottish Hospital Inpatients' Statistics (SHIPS) which links all NHS hospital admissions in Scotland based on ICD coding (Kendrick *et al* 1993). Virtually all children with IBD in Scotland are hospitalised at least once (Barton *et al* 1989b) and hence identification of a geographically defined cohort from the SHIPS database is likely to give a representative sample of juvenile-onset IBD children in Scotland. It was encouraging to note that some of the deficiencies in monitoring of growth and development variables in Scotland reported previously by my predecessor (Barton *et al* 1989a) had improved in the more recently diagnosed cohort. However, the monitoring was far from perfect and deficiencies in recording height, bone age and sexual development were still not uncommon. Some aspects of morbidity showed evidence of improvement in the more recently diagnosed cohort, such as the frequency of resectional surgery and the duration of hospitalisation in juvenile onset CD patients. This coincided with more frequent use of systemic steroids, immunosuppressives and elemental diet in the more recently diagnosed CD cohort. However, there was no significant improvement in the frequency of height and weight retardation between the previous and the more recently diagnosed cohort. The prospect of the juvenile-onset IBD patients achieving their projected adult height was quite good and this was shown by height measured at the time of the interview when most of the 1984-88 cohort were adults. This was also the conclusion of a previous report from my institution which specifically looked at long-term outcome of growth and developmental delay in juvenile-onset IBD patients (Ferguson *et al* 1994). A Canadian study reached a similar conclusion (Griffiths *et al* 1993).

These two studies in adolescents confirm the profound nutritional effects of CD and complement the studies in the adults. In fact, they identify this group as being particularly vulnerable to the effects of nutritional depletion as reflected by osteopenia and growth retardation. It is encouraging to note that monitoring of growth and developmental parameters is improving in this group, but further awareness of the problem is needed to ensure the ideal of universal monitoring which is essential in this group.

10.8. Conclusions and implications

The aim of this thesis was to explore the direct effects of IBD on specific aspects of nutrition. A number of previously unrecognized effects of disease on nutrition were identified. The study of bone mineralisation in newly diagnosed IBD clearly demonstrates osteopenia in CD, but not in UC. The influence of the confounding variables discussed mask this difference in long-standing disease. Small numbers make it difficult to rule out type II errors in analysing the effects of smoking and menstrual status. Change in body composition, particularly a preferential loss of fat over lean was detected in parallel with osteopenia. The metabolic basis of this finding needs further investigation. Adolescents were identified as particularly vulnerable to nutritional problems associated with CD, and nutritional monitoring in this group, though improving, needs to be universal. Bedside methods of measurement of body composition are needed to explore these issues further in large, preferably community-based cohorts, and a simple, user-friendly bioelectrical impedance analysis machine shows promise as a suitable method. Finally the results provide further evidence that CD and UC are different diseases - CD is a systemic disease with widespread immunological and nutritional problems, whereas UC is limited to the mucosa with few systemic effects.

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SECTION III

Immunological Studies In Inflammatory Bowel Disease

Chapter XI

LABORATORY METHODS FOR OBJECTIVE ASSESSMENT OF COMPONENTS OF INTESTINAL INFLAMMATION

11.1.Objective clinical assessment of gut inflammation and injury

In order to study the gut immune system in clinical situations, a range of components should be assessed. As discussed in chapter II, tests on components of the systemic immune system such as blood antibodies and circulating cells and cytokines are generally useless as indices of mucosal immunity at gut level. Published evidence from our laboratory has shown that data based on analysis of faeces is highly misleading (Ferguson *et al* 1995). My predecessors in the gastrointestinal laboratory have established that analysis of the clear fluid obtained after whole gut lavage is a powerful method of assessing intestinal immunity, inflammation and gut losses of protein and blood (O'Mahony *et al* 1990, Brydon *et al* 1992 and 1993). Whole gut lavage fluid (WGLF) analysis has been reviewed in chapter II.

11.2. Whole gut lavage

The patients drank an isotonic polyethylene glycol-electrolyte fluid (Klean-prep, Norgine Ltd., Headington, Oxford, UK) at a rate of 250mL every 15 minutes, supervised by an experienced nurse (Choudari *et al* 1993). The first completely clear fluid specimen passed per rectum was collected for analysis. Aliquots of unfiltered, unprocessed specimens were stored at -70°C until assayed for GE. The rest of the specimen was filtered through glass microfibre filters (Whatman GF/A, Whatman International Ltd, Maidstone, England) with a pore size of 1.6μm and aliquots were stored without further processing for assay of free GE. Other aliquots of the filtered specimen were processed by the addition of protease inhibitors. The following processing reagents were added to the filtered fluid with mixing after each addition : soybean trypsin inhibitor in phosphate buffered saline (PBS) (80 μg/mL), sodium ethylenediaminetetraacetic acid in PBS (15 mmol/L),

phenyl methyl sulphonyl flluoride in 95% ethanol (2 mmol/L), sodium azide (1 mmol/L) and newborn calf serum (5% volume). Aliquots of processed WGLF were stored at -70°C and later assayed for IgG by enzyme-linked immunosorbent assay (Choudari *et al* 1993), and for cytokines as described later in this chapter.

The study protocol was approved by the Medicine Subcommittee of the Lothians Area Ethics of Research Committee.

11.3. Assessment of neutrophils in whole gut lavage fluid

11.3.1. Cytology

Each specimen of WGLF (25mL) was centrifuged at 850*g* for 10 min. The supernatant was discarded and the pellet resuspended in 5mL Hanks' balanced salt solution (Sigma Chemical Co. Ltd, Poole, UK). This was layered on top of a discontinuous density gradient using Histopaque 1119 and 1077 (Sigma). After centrifugation for 30 min at 700*g* the 2 resultant layers were harvested separately. The suspensions were washed with Hanks' balanced salt solution and the pellets resuspended in 1 mL. Cell counts were performed with a haemocytometer, and slides of the 2 suspensions were made using a Cytospin cytocentrifuge (Shandon Southern Products Ltd, Runcorn, UK) operated for 10 min at 90*g*. The slides were air-dried and then fixed in methanol before being stained by the May-Grunwald-Giemsa protocol. Slides were examined with an Ortholux II microscope (Leitz, Wetzlar, Germany), and the cell types present were recorded. Additionally the number of neutrophils in the preparation was subjectively graded as a) few or none, b) moderate numbers or c) very many. Slides were also immunostained using monoclonal antibodies for anti-CD3 (Scottish Antibody Production Unit, Carluke, UK), anti-L26 (DAKO Ltd, High Wycombe, UK) and anti-CD68,KP1 (DAKO) to detect T lymphocytes, B lymphocytes and macrophages. The three-stage streptavidin-biotin-alkaline phosphatase technique was used (Hsu *et al* 1981).

11.3.2. Granulocyte elastase (GE)

i)Total GE: The highly specific substrate for GE, L-Pyroglutamyl-L- prolyl-L-valine-p-nitroanilide (Kramps *et al* 1983), was obtained from Quadratech (Epsom, UK) and was dissolved in dimethylsulphoxide and diluted four times in water to give

a final concentration of 2 mmol/L. Approximately 1μL of Triton X-100 (Sigma) was mixed into 500μL of thawed, unfiltered, unprocessed WGLF {the precise amount of Triton is not critical (Kramps *et al* 1980)}. Each sample was sonicated at 0°C for 1 min 3 times using a Rapidis A180G sonicator (Ultrasonics Ltd., Shipley, UK) to disrupt the cells present and release the enzymes contained in the granules. 200μL of sample was added to 200μL of buffer (Tris 0.1 mol/L, NaCl 0.96 mol/L, pH 8.3) and the mixture was incubated at 37°C. 200μL of substrate at 37°C was added and after exactly 3 min the reaction was stopped with 200μL of 20% acetic acid. Blank tubes were run for all samples in the same way, but with the acetic acid added before the substrate. Absorbance was measured at 405nm and GE concentration calculated using the formula provided by Quadratech: (test A_{405} - blank A_{405}) x 2310 nkat/L. A crude human granulocyte extract was prepared by a modification of the method of Kramps *et al* 1980 and was included as a standard.

ii)Free: Thirtynine patients who had total GE detectable by the above assay also had GE measured in a filtered, unprocessed sample. The method described above was used without the sonication step. The process of filtration removed the granulocytes and hence any GE detectable in this specimen represented free or non-particulate GE.

11.4.Pro-inflammatory cytokines

11.4.1. Interleukin-1β (IL-1β)

Quantitative measurement of IL-1β in stored WGLF was carried out using a commercial, high sensitivity IL-1β ELISA kit (Cistron Biotechnology, New Jersey, USA). WGLF which had been filtered and processed by the addition of protease inhibitors, as described above, was used. The ELISA procedure was a four-stage test carried out in microtitration wells which were supplied coated with monoclonal antibody to IL-1β. In the first stage, the samples or IL-1β standards were added to the wells and incubated. IL-1β present in the sample was bound to the solid-phase anti-IL-1β. If IL-1β was not present, all sample components were removed in the subsequent washing step. In the second stage, polyclonal rabbit anti-IL-1β was added to the wells. The antibody was bound specifically to IL-1β bound to the solid phase. In the third stage, goat anti-rabbit IgG conjugated to horseradish peroxidase

enzyme was added to the wells. The conjugate bound specifically to any bound rabbit IgG. Between stages, washing steps removed any unbound components from the wells. In the fourth stage, the enzyme-substrate system was added to the wells. The substrate was oxidised in the presence of conjugate, resulting in a coloured end product. Acid was added to stop the reaction and fix the colour. The colour intensity was measured with a microtitration plate reader at a wavelength of 450nm. A standard curve was constructed and test results read from it. The lower limit of detection by this assay was 4pg/mL.

11.4.2. Interleukin-8 (IL-8)

Stored WGLF which had been filtered and processed by the addition of protease inhibitors was used. A commercially available quantitative sandwich enzyme immunoassay technique was used (Quantikine, R&D Systems, Minneapolis, USA). The 96-well polystyrene microtitre plate was supplied coated with a murine monoclonal antibody against IL-8. Recombinant human IL-8 standard and samples were pipetted into the wells and any IL-8 present was bound by the immobilised antibody. After washing away any unbound proteins, a polyclonal antibody specific for IL-8 conjugated to horseradish peroxidase was added to the wells to `sandwich' the IL-8 immobilised during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of IL-8 bound in the initial step. The colour development was stopped and the intensity of the colour development measured with a microtitration plate reader at a wavelength of 450nm. A curve was prepared plotting the optical density versus the concentration of IL-8 in the standard wells. By comparing the optical density of the samples to this standard curve, the concentration of IL-8 in the unknown samples was then determined. The lower limit of detection of IL-8 by this assay was 18 pg/mL.

11.4.3. Interferon-γ (IFN-γ)

WGLF that was filtered and processed by the addition of protease inhibitors was used. IFN-γ was assayed by an in-house ELISA. Immulon 2 plates (Dynatech Laboratories Inc., Virginia) were coated with a monoclonal anti-IFN-γ antibody (Chromogenix AB, Molndal, Sweden) and incubated overnight at 4°C. The wells

were then blocked with 1% bovine serum albumin (Sigma) in PBS for 1 hour. The 1st British Standard for human leukocyte IFN-γ (NBSB, NIBSC, Potters Bar) was used as a standard. Serial dilutions of standard and 1/2 dilutions of samples were added to the wells and incubated at room temperature for 3 hours. Washing was carried out between all steps. Biotinylated monoclonal antibody (Chromogenix) against IFN-γ was added and incubated overnight at room temperature. ExtrAvidin alkaline phosphatase conjugate (Sigma) was added which bound to any biotinylated monoclonal antibody present, and incubated for 1 hour, followed by development with *p*NitroPhenylPhosphate substrate (Sigma) in 10% Diethanolamine buffer. The plate was read at 405 nm, and the sample results calculated from the standard curve. The lower limit of detection was 0.64 IU/mL.

11.4.4. Interleukin-6 (IL-6)

Filtered processed WGLF was used. IL-6 was assayed by an in-house ELISA. Costar high binding capacity plates (Costar UK Ltd, High Wycombe) were coated at room temperature for 2 hours with polyclonal sheep antibody to human IL-6 (CLB, Amsterdam). After washing, they were blocked with 5% bovine serum albumin (Sigma) in PBS overnight. The standard used was recombinant DNA human type IL-6 reference preparation (NBSB, NIBSC, Potters Bar). It was serially diluted in 50% PBS/Tween 20/ABS, 50% polyethylene glycol with protease inhibitors to maintain compatibility with the samples. Samples(1:2 dilutions in PBS/Tween 20/ABS) were added and the plates incubated at room temperature for 2 hours. Washing steps were carried out between each reagent. Murine monoclonal antibody to human IL-6 (CLB) was then added and incubated for 2 hours at room temperature. Next biotinylated sheep anti-mouse Ig, species specific whole antibody (Amersham International Plc, Amersham) was added and incubated for 1 hour at room temperature. ExtrAvidin alkaline phosphatase (Sigma) and *p*NitroPhenylPhosphate substrate were added in two subsequent steps as described under IFN-γ. After reading at 405nm, a standard curve was constructed and used to obtain test results. The lower limit of detection was 20 pg/mL.

11.5. Growth factor peptides

11.5.1. Insulin-like growth factor -1 (IGF-1)

Preliminary experiments established that IGF-1 could be detected in WGLF samples that had been filtered and processed by the addition of protease inhibitors, but not in fluid to which protease inhibitors had not been added. Hence stored WGLF samples which had been filtered and processed were used. A recently available commercial two-site immunometric assay (Octeia® IGF-1 IEMA, IDS Ltd, Tyne and Wear, UK) for the quantitative determination of IGF-1 was used. The method incorporated a sample pre-treatment to avoid interference with IGF-1 binding proteins.

The lavage samples were incubated briefly with the releasing agent to inactivate binding proteins, and then diluted for assay. The polystyrene microtitre wells were supplied coated with a purified sheep polyclonal anti-IGF-1. The pre-treated diluted samples were then incubated, together with horseradish peroxidase-labelled monoclonal anti-IGF-1, in antibody coated wells for 2 hours at roam temperature. The wells were washed and a single component chromogenic substrate (a formulation of tetramethylbenzidine) was added to develop colour. The absorbance of the stopped reaction mixture was read in a microtitre plate reader at 450nm, the colour intensity developed being directly proportional to the amount of IGF-1 present in the sample. A set of 6 calibrators were supplied as standards and two controls for quality control of assays. As the concentrations of IGF-1 in the samples were low, the absorbence of the calibrators were plotted against the concentration of low calibrator values on a linear-linear graph paper. Results for the unknowns were read directly from the curve. The lower limit of detection of the assay was 1.1μg/mL.

11.5.2. Transforming growth factor β_1(TGF-β_1)

This was measured in stored, filtered, processed WGLF. A commercial TGF-β_1 ELISA kit was used (Predicta® TGF-β_1 kit, Genzyme diagnostics, Cambridge, MA, USA). The Predicta TGF-β_1 ELISA kit contains a 96-well microtitre plate with immobilized mouse monoclonal antibody to TGF-β_1. Activation of TGF-β_1 was required prior to its measurement *in vitro*. The samples, standards and controls were diluted by a factor of 2.17 and acidified using Hcl for one hour. Following

incubation, acidified samples and standards were neutralized to pH 7.0-7.4 with NaOH, and were ready to be assayed.

Activated samples, standards or controls were added to each test well and incubated to allow any TGF-β_1 present to be bound by antibodies on the microtitre plate. The wells were then washed and a direct-labelled Horseradish peroxidase-conjugated polyclonal anti-TGF-β_1 was added which bound to the captured TGF-β_1 during incubation. After washing, a substrate solution was added to the wells, producing a blue colour in the presence of peroxidase. The colour reaction was then stopped by the addition of acid which changed the blue colour to yellow. The absorbence of each well was read at 450nm and a standard curve was constructed to quantitate TGF-β_1 concentrations in the controls and samples. The lower limit of detection of the assay was 50 pg/mL.

11.6. Statistical Methods

These are discussed in the results sections of individual chapters that follow. Non-parametric statistics were used as the data were not normally distributed. The Minitab statistical software (release 8.2) was used for all analysis.

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Chapter XII

INVESTIGATION OF NEUTROPHIL MIGRATION INTO THE GUT BY CYTOLOGY OF WHOLE GUT LAVAGE FLUID.

12.1. Introduction

Whole gut lavage is a relatively new technique for non-invasive investigation of gut immunity (O'Mahony *et al* 1990, O'Mahony *et al* 1991), and can be used for research on mucosal immunoglobulins and antibodies, to measure gut losses of blood and protein, and for studies of local immunoregulatory and pro-inflammatory cytokines. It has been known for several years that tumour cells may be found by cytological investigation of WGLF in patients with gastro-intestinal cancers (Brandt *et al* 1989, Rozen *et al* 1990, Gordon *et al* 1991, Wuerker *et al* 1993).

Experiments with intravenously administered mixed leukocyte preparations labelled with ^{111}In or ^{99m}Tc have shown that these cells rapidly localise in inflamed bowel, migrate into the bowel lumen, and are excreted in faeces (Saverymuttu *et al* 1983, Scholmerich *et al* 1988). Quantitation of the faecal excretion of these radiolabelled cells has been used to assess disease activity in patients with IBD (Saverymuttu *et al* 1983b, Saverymuttu *et al* 1986, Pullman *et al* 1988). It seemed probable that leukocytes in the gut lumen would also be detectable by tests on WGLF, and if so, a simple, non-isotopic method to research this aspect of gut inflammation could be developed.

In this chapter, I have assessed how luminal neutrophilia relates to the type and distribution of IBD, and to disease activity in a series of well characterised patients with IBD and other conditions. The cells in WGLF were counted and examined microscopically by Mrs Louise Handy.

12.2. Subjects and methods

12.2.1. Subjects

WGLF samples from 56 patients were used for cytological assessment. Each patient had the gastrointestinal tract assessed by an appropriate combinations of upper endoscopy, colonoscopy, jejunal biopsy, other histology and

contrast radiology. Laboratory investigations performed included determinations of haemoglobin, white cell count, ESR, albumin and C-reactive protein. All medications and diet at the time of lavage were recorded.

Thirteen patients were immunologically normal, with anatomically normal GI tracts and were used as controls. Twenty-nine patients had IBD. They included: (a) 12 active Crohn's disease (CD): 5 colonic CD (including additional small bowel involvement), 4 small bowel CD, 3 other CD (e.g. perianal); (b) 10 inactive CD: 5 colonic CD, 2 small bowel CD, 3 other CD; (c) 3 active ulcerative colitis (UC); (d) 4 UC in remission. Of the UC patients, 4 had pancolitis, one had distal colitis and two had proctitis. IBD was classified as active when the WGLF IgG concentration was greater than 10μg/mL (see below). Six patients had other benign, upper GI tract diseases, and seven had colonic diseases other than IBD (radiation colitis (2), benign polyps (2), infectious colitis, diverticulitis, stercoral ulcer). An eighth patient, with colon carcinoma complicating long-standing inactive UC, was classified as being in this "miscellaneous colonic disease" group.

12.2.2. Lavage protocol

The patients drank an isotonic polyethylene glycol-based fluid (Klean-prep, Norgine Ltd., Headington, Oxford, UK), supervised by an experienced nurse, as described in chapter XI. The first completely clear fluid specimen passed per rectum was collected for analysis. Cytological assessment was performed immediately after collection as described below. Aliquots of the specimen were also filtered, processed by the addition of protease inhibitors, stored at -70°C and later assayed for IgG by enzyme-linked immunosorbent assay (Choudari *et al* 1993).

The protocol was approved by the Medicine Subcommittee of the Lothian's Area Ethics of Research Committee.

12.2.3. Disease activity in IBD patients

One of the advantages of using WGLF for IBD research is that disease activity can be graded by measurements of IgG concentration in WGLF. This has previously been validated by Choudari *et al* 1993 who found that the Crohn's disease activity index (CDAI) for CD and the Powell Tuck index (PTI) for UC correlated well with WGLF IgG concentration. In the same series of patients, an experienced physician's global assessment was also very reproducible and

correlated with CDAI or PTI, and with WGLF IgG concentration (Ferguson *et al* 1994a).

For the purposes of analysis and statistical comparisons, I have classified IBD patients as active when the IgG concentration in WGLF was >10 μg/ml, and inactive when IgG level was ≤10 μg/ml. In the present study, I have again compared values for WGLF IgG concentration in the 29 IBD patients with a physician's overall clinical decision as to whether the disease was active or inactive (based on symptoms, clinical signs and results of haematological and biochemical blood tests). The categorisation as active or inactive based on IgG concentration was the same as the clinical classification in all cases. The conventional laboratory indices of disease activity in the IBD patients (serum albumin, CRP, white cell count and ESR) along with the physician's clinical classification into active or inactive disease are given in table 12.1.

Table 12.1. Conventional laboratory markers of disease activity in 29 IBD patients and physician's global assessment

IBD group	Serum albumin (g/L)median & range	C-reactive protein(mg/dl)median & range	White cell count ($x10^9$/L) median & range	ESR (mm/1st hr)median & range	No. classified as active disease by physician's global assessment
ACTIVE CD					
Colonic (n=5)	37 (35-42)	7.6 (<1.5-16.5)	11.6 (9.5-14.5)	46 (20-89)	5
Small bowel (n=4)	38 (23-41)	<1.5	8.5 (6.0-10.2)	34 (7-74)	4
Others (n=3)	40 (22-43)	1.5 (<1.5-2.0)	7.3 (5.4-7.8)	27 (4-44)	3
INACTIVE CD					
Colonic (n=5)	42 (34-45)	<1.5	8.7 (6.5-15.9)	2 (1-13)	0
Small bowel (n=2)	38,41	<1.5	5.1,9.4	4,12	0
Others (n=3)	42 (40-45)	<1.5	5.8 (5.5-6.2)	27 (2-100)	0
ACTIVE UC (n=3)	39 (39-43)	<1.5	8.4 (7.5-9.3)	13 (2-24)	3
INACTIVE UC (n=4)	45 (41-46)	<1.5	6.6 (5.4-7.2)	12 (6-40)	0

12.2.4. Cytology preparations

This has been described in details in chapter XI.

12.2.5. Statistical analysis

Comparisons between different groups of subjects were done by the Mann-Whitney U test and χ^2 test with Yates correction. p values <0.05 were considered significant.

12.3. Results

12.3.1. WGLF cytology

During initial attempts at cytological examination of WGLF, either samples filtered through 2 layers of surgical gauze before centrifugation, counting and cytocentrifugation, or samples centrifuged and layered onto one high density separation medium only were used. These methods produced slide preparations which contained considerable amounts of debris and many bacteria, in addition to variable numbers of cells. Modification of the technique, as described in `Methods' section by using two density separation media, greatly reduced bacterial contamination and debris.

It was anticipated that neutrophils and lymphocytes would layer separately at the two interfaces during gradient centrifugation, but this was not the case. Most cells were recovered from the upper of the two layers, and the cell types seen in the top layer were also seen, though in fewer numbers, in the bottom layer. The data reported below are total cell counts for both layers added together, and cytological descriptions for both layers have also been combined.

The cells present were neutrophils, squamous cells, enterocytes, eosinophils, macrophages, erythrocytes, and, very occasionally, lymphocytes; cells from a patient with active CD are illustrated in Figure 12.1. Some samples contained yeasts, and bacteria were ubiquitous.

12.3.2. Cell counts

Figure 12.2 shows the counts for the different disease groups. Specimens with many neutrophils seen on cytospin slides are highlighted. In normal subjects

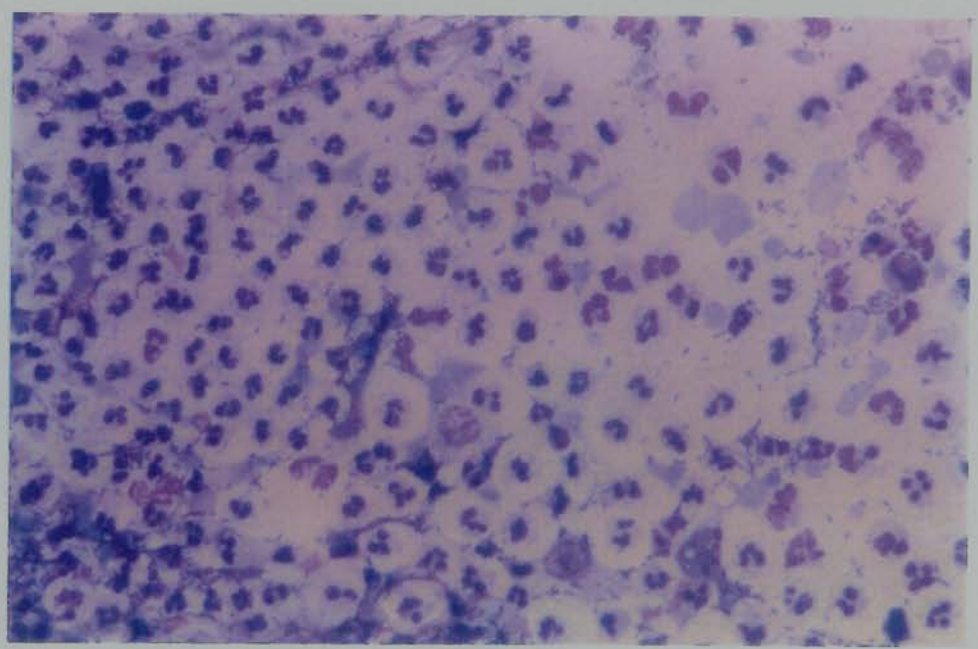

Figure 12.1. Typical appearance of WGLF cytology from a patient with Crohn's disease, showing neutrophils, squamous cells and enterocytes. Magnification X 40. May-Grunwald-Giemsa stain.

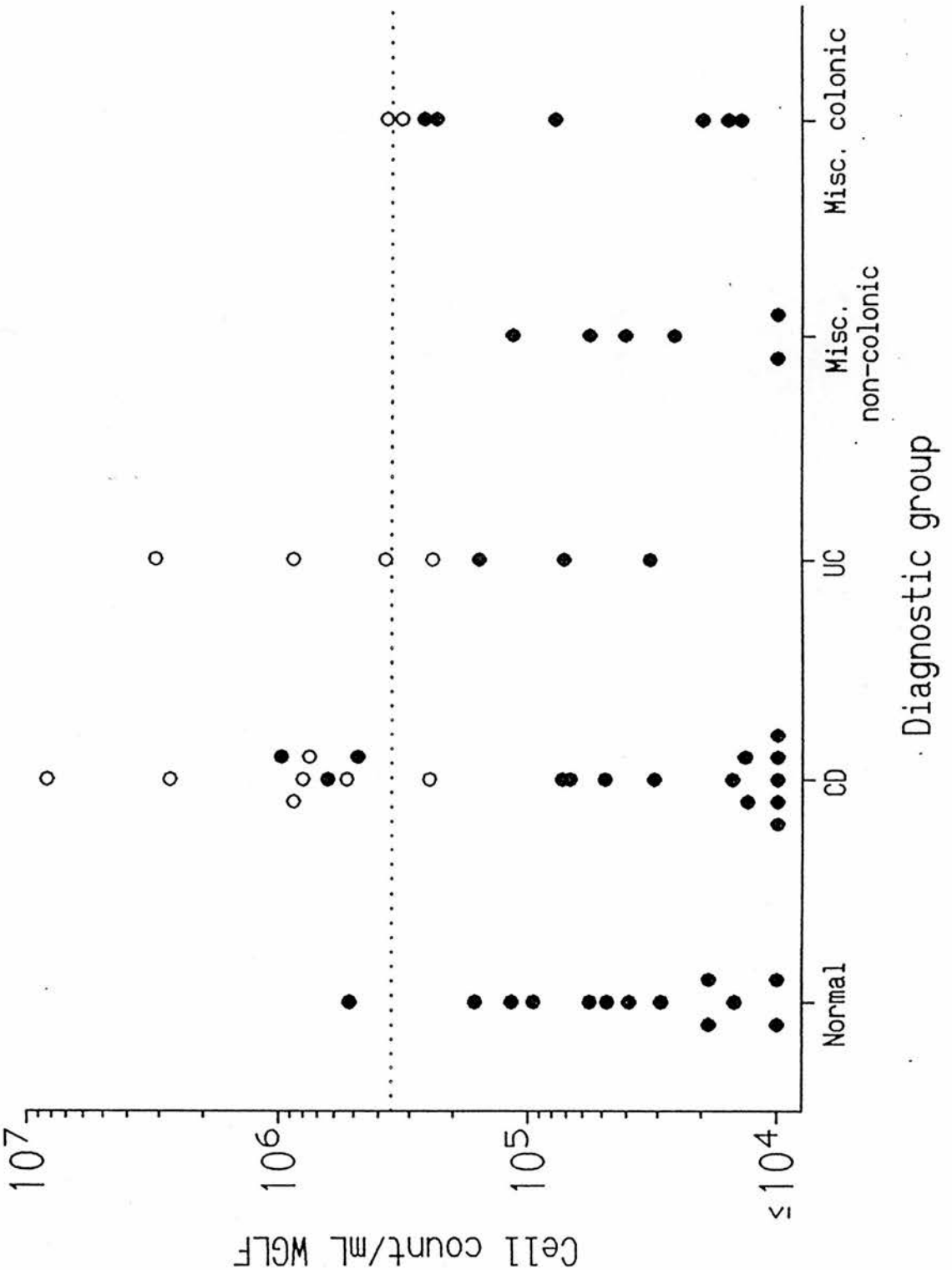

Figure 12.2. Number of cells/mL WGLF plotted against diagnostic group, highlighting cases with many neutrophils. O = many neutrophils seen, • = all other cases. ····· shows the count/mL equal to the mean + 2SD of the normal group.

the mean + 2SD of the cell count was 3.7 x 10^5/ml WGLF, and this value has been taken as the upper limit of the reference range. In the single normal subject with a cell count above this level, microscopy showed that these were predominantly squames. Nine of the 22 patients with CD and three of the seven with UC had high cell counts, as did one of the 14 other diseased patients (cell count 3.8 x 10^5/mL, in WGLF from a patient with infective colitis). Information on the cell types in WGLF from IBD patients are given below. Two of the 14 other patients had many neutrophils in cytospins of WGLF, a patient with infective proctitis (with high WGLF IgG also) and the patient with colonic carcinoma and long-standing inactive UC.

12.3.3. Cell counts in IBD according to disease activity and anatomy of involvement

Figure 12.3 shows the cell counts in WGLF from IBD patients, subdivided into UC, colonic CD, small bowel CD and CD with other distribution. Results are presented in relation to IgG concentration in WGLF, the objective measure of disease activity. Table 12.2 summarises the values for selected subgroups of patients, together with the results of statistical analyses. In general, patients with active IBD had higher counts than those with inactive disease ($p<0.05$) , and despite the relatively small numbers studied, differences were significant for active IBD when compared with normals ($p < 0.05$). However the subgroup with active small bowel CD did not conform ; cell counts were in the normal range in three of the four patients concerned (Figure 12.3).

12.3.4. Cell types in IBD according to disease activity and anatomy of involvement

Cytospin preparations revealed many neutrophils in 6 of the 10 specimens from colonic CD patients ($p < 0.01$ when compared with normals), from one of 12 other CD patients (not significant) and from 4 of the 7 samples from UC patients ($p< 0.02$).

When these data are further examined in relation to disease activity, a clear influence of site of IBD emerges. Very many neutrophils were seen in cytospin preparations from all three patients with active UC, all five with active

Table 12.2. Cell counts in WGLF in different IBD groups and normals.

Diagnostic group	n	cell count/mL median (range)	p
Normals	13	3.9×10^4 (1×10^4 - 5.2×10^5)	
Active IBD	15	5.4×10^5 (<1×10^4 - 8.4×10^6)	<0.05 Vs normals
Inactive IBD	14	4.1×10^4 (<1×10^4 - 8.8×10^5	<0.05 Vs active IBD
Active CD	12	6.5×10^5 (<1×10^4 - 8.4×10^6)	NS Vs normals
Inactive CD	10	2.3×10^4 (<1×10^4 - 6.4×10^5)	< 0.05 Vs active CD
Active CD: colonic	5	8.1×10^5 (5.4×10^5 - 8.4×10^6)	<0.01 Vs normals
small bowel	4	4.1×10^4 (<1×10^4 - 2.7×10^6)	NS Vs normals
other	3	7.3×10^4 (<1×10^4 - 9.8×10^5)	NS Vs normals
Inactive CD:colonic	5	4.9×10^4 (1.3×10^4 - 4.8×10^5)	NS Vs normals
small bowel	2	<1×10^4 , 6.4×10^5	
other	3	<1×10^4 (<1×10^4 - 3.1×10^4)	NS Vs normals
Active UC	3	3.8×10^5 (2.4×10^5 - 3.1×10^6	< 0.05 Vs normals
Inactive UC	4	1.2×10^5 (3.2×10^4 - 8.8×10^5).	NS Vs active UC

colonic CD, and in only one of the 4 patients with active small bowel CD, a patient with ileal disease complicated by an abscess who was also on high dose nonsteroidal anti-inflammatory drugs for ankylosing spondylitis (table 12.3). There was no relationship between disease distribution and neutrophils in cytospin preparations from patients with inactive IBD.

12.3.5. Other cell types.

Eosinophils were seen in preparations from 3 of the 22 CD cases, 3 of the 7 UC patients and in the patient with colonic carcinoma complicating UC; in all of these, neutrophils were also present in the WGLF. Enterocytes and squamous cells were commonly seen in slides both with and without neutrophils. Erythrocytes occurred occasionally in slides from IBD patients, and macrophages were seen occasionally in a variety of clinical conditions. Lymphocytes were very rarely observed and occurred in low numbers, mostly in IBD cases. Immunostaining of the few specimens which contained lymphocytes confirmed the presence of B cells, plasma cells, and T cells, but the general paucity of lymphocytes was striking. No malignant cells were seen.

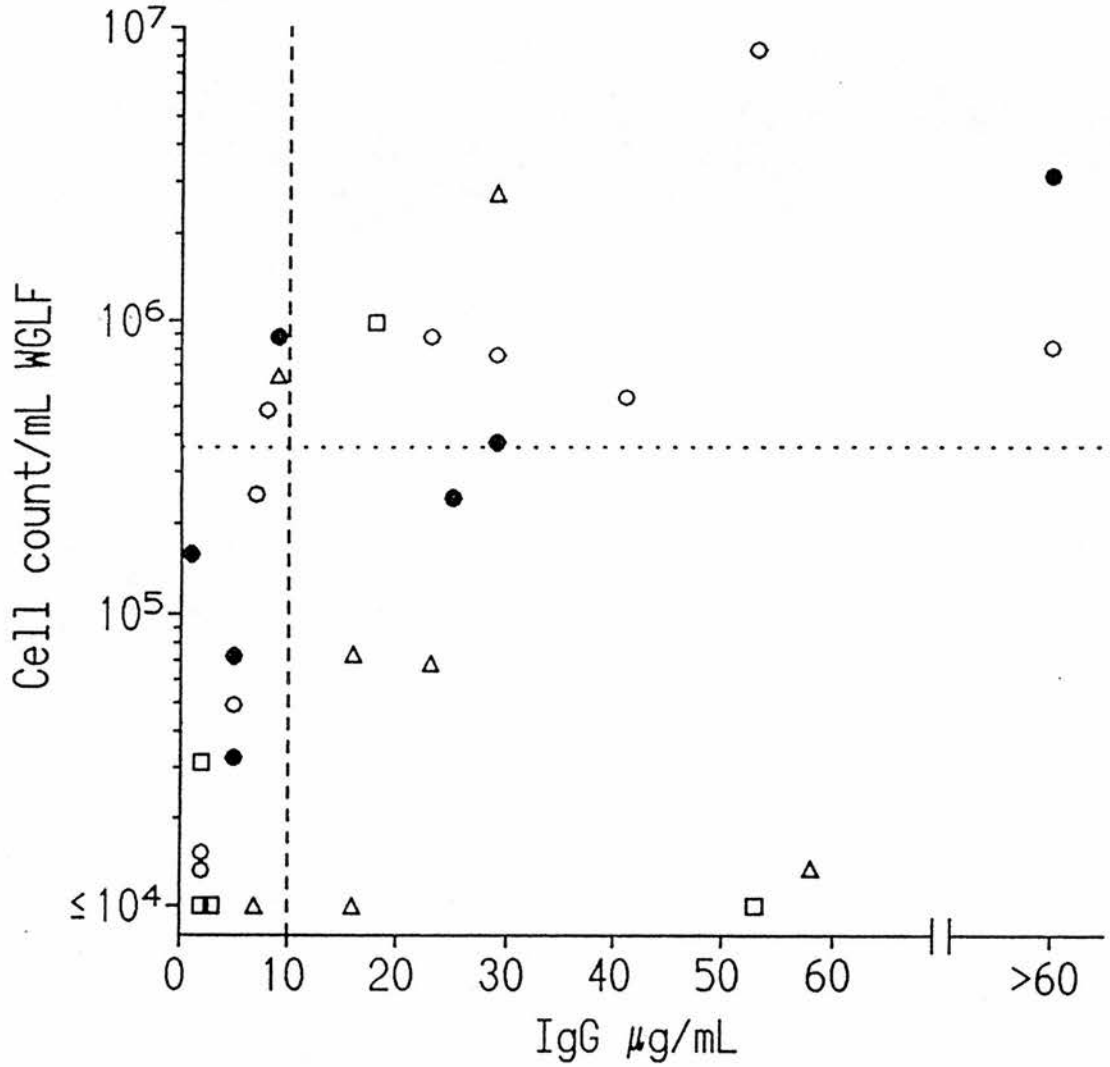

Figure 12.3. Cell count/mL WGLF plotted against WGLF IgG concentration for IBD cases. o = colonic CD, Δ = small bowel CD, □ = other CD, • = UC. ····· shows the count/mL equal to the mean + 2SD of the normal group and - - - - - shows the upper limit of normal for IgG concentration.

Table 12.3. Presence of neutrophils in different diagnostic groups.

Diagnostic group	n	none/ few	some	very many
Normal	13	13	0	0
Active IBD:				
Colonic CD	5	0	0	5
Small bowel CD	4	3	0	1#
Other CD	3	2	1	0
UC	3	0	0	3
Inactive IBD:				
Colonic CD	5	3	1	1
Small bowel CD	2	2	0	0
Other CD	3	3	0	0
UC	4	2	1	1
Other non-colonic disease	6	5	1	0
Other colonic disease	8	5	1*	2**

complicated by abscess and long term high dose NSAID for ankylosing spondylitis.

* diverticulitis

** infective colitis and colon carcinoma complicating UC

12.3.6. Conclusion

Cytology of WGLF is a valuable technique for investigating neutrophil migration into the gut lumen. Luminal neutrophilia is a feature of active UC and active colonic CD, but not small bowel CD.

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Chapter XIII

INVESTIGATION OF NEUTROPHIL MIGRATION INTO THE GUT BY ASSAY OF GRANULOCYTE ELASTASE IN WHOLE GUT LAVAGE FLUID

13.1. Introduction

In the previous chapter, I described cytological examination of whole gut lavage fluid (WGLF) for investigation of neutrophil migration into the lumen of the gut. Cytology is, however, time consuming, requires expert cytologists and cannot be performed on stored samples. The granules of polymorphonuclear leukocytes contain enzymes such as elastase (Janoff 1972) and myeloperoxidase (Borregaard 1988). Granulocyte elastase (GE) can be detected in faeces from patients with gut inflammation (Bohe 1987, Adeyemi *et al* 1992), and so it is likely that assay of GE could supplement or replace microscopy for the detection and quantification of intestinal granulocytes in the gut lumen.

In this chapter I have described the study of gut luminal neutrophils by assay of GE in WGLF from a large series of well characterised patients with IBD and other conditions. I will also examine how luminal neutrophilia relates to the type and distribution of IBD, to disease activity, and to the effects of corticosteroid and antibiotic therapy.

13.2. Subjects and methods

13.2.1. Subjects

WGLF samples from 203 patients were analysed, in whom the gastrointestinal tract was assessed by an appropriate combination of upper endoscopy, colonoscopy, jejunal biopsy, other histology and contrast radiology. All medications and diet at the time of lavage were recorded. Laboratory investigations performed included determinations of haemoglobin, white cell count, ESR and C-reactive protein. IBD was classified as active when the WGLF IgG concentration was greater than 10µg/mL.

Thirty-one patients had a normal gastrointestinal tract and acted as controls. Forty-three patients had Crohn's disease (CD) and 25 had ulcerative colitis (UC). Sixty-three patients had miscellaneous non-colonic gastrointestinal diseases and 41 had colonic diseases other than IBD. Further details can be found in Table 13.1. A woman with colonic carcinoma complicating long-standing, inactive UC was included in the miscellaneous colonic diseases group.

In 47 of the above patients, cytological assessment was performed on the first clear specimen immediately after collection.

Table 13.1. Details of 203 subjects studied for granulocyte elastase

Diagnostic group	n (M:F)	IBD Active:inactive (WGLF IgG >10:≤10 μg/mL)	Comments
Normals	31 (14:17)	N/A	Patients with normal GI tract & volunteers from other studies
CD	43 (18:25)	23:20	17 colonic 13 small bowel only 13 others (perianal, orogenital, ileostomy)
UC	25 (10:15)	10:15	11 pancolitis 5 distal colitis 8 proctitis 1 ileostomy
Miscellaneous noncolonic	63 (23:40)	N/A	10 upper GI lesions 27 Fe deficiency anaemia 26 small bowel lesions
Miscellaneous colonic	41 (20:21)	N/A	10 radiation colitis 6 colonic carcinoma 1 ischaemic colitis 1 infective colitis 1 colon carcinoma complicating long-standing inactive UC 19 others (e.g. polyps, diverticulitis, diverticular disease)

13.2.2. Methods

Unfiltered, unprocessed WGLF was used to assay GE using a specific enzyme-substrate reaction. This is described in chapter XI. The assay was developed by Mrs Louise Handy.

13.2.3. Statistical analysis

Correlation coefficients were calculated using the Spearman Rank Correlation test. Comparisons between different groups of subjects were carried out using the Mann-Whitney test and χ^2 test with Yates correction; p values <0.05 were considered significant.

13.3. Results

13.3.1. Disease activity in IBD patients

In the present study, I again compared values for WGLF IgG concentration in the 68 IBD patients with a physician's overall clinical decision as to whether the disease was active or inactive (based on symptoms, clinical signs and results of haematological and biochemical blood tests). The categorisation as active or inactive based on IgG concentration (>10μg/mL or ≤10μg/mL) was the same as the clinical classification in 65 of the 68 cases. Of the three with discrepant results, two had severe UC and were on intensive medical treatment with intravenous steroids; they were clinically graded as having active disease whereas WGLF IgG levels were 9 and 6μg/mL. The third patient had colonic CD, was on maintenance oral steroid therapy, had few symptoms and normal inflammatory parameters in blood apart from a raised ESR. She was clinically graded as inactive but her WGLF IgG concentration was 16μg/mL.

For the purposes of analysis and statistical comparisons in this report, we have used the objective measure, WGLF IgG concentration: IBD active when IgG >10 μg/ml, and inactive when IgG ≤10 μg/ml.

13.3.2. WGLF GE measurement

The GE assay technique is based on the method used for measurement of elastase in granulocyte extracts from whole blood. Possible interference by high polyethylene glycol concentrations was excluded by comparing values for blood granulocyte suspensions in balanced buffer and in surrogate WGLF preparations; no difference was detected. The handling of the WGLF sample after collection was also of importance. For immunoassays, including IgG measurements, WGLF samples must be filtered and treated with a series of protease inhibitors. Such

specimens have, of course, been depleted of cells, including granulocytes, at the filtration stage. Also, in the presence of protease inhibitors GE cannot be detected by the method used. Thus, total GE measurements were made on WGLF specimens untreated apart from sonication. GE concentrations of below 39 nkat/L were considered to be below the reliable limit of detection of the assay. The between-assay coefficient of variation was 6%. Free GE was however measured in specimens after filtration, which removed the neutrophils (10-15μm in diameter); hence these specimens were not sonicated.

13.3.3. Relationship between cell count and GE concentration in WGLF

Figure 13.1 demonstrates the relationship between WGLF cell counts and GE concentrations in the 47 patients studied by both methods. The correlation for the whole series, for CD and for UC patients are all highly significant (all cases $r = 0.80$, $p < 0.001$; CD $r = 0.79$, $p < 0.001$; UC $r = 0.98$, $p = 0.001$). There were 33 specimens in which GE was less than the 39 nkat/L detection limit.

13.3.4. GE concentrations in WGLF

Figure 13.2 shows GE concentrations for the specimens by disease group. Of the 31 normals (median <39, range <39-113 nkat/L) GE was detectable in only 4 samples (values of 60,74,74,113 nkat/L). I have therefore analysed results in the various patient groups as absolute GE values, and as the number of patients with any detectable GE; values >100 nkat/L WGLF are considered abnormally high. Detection of GE was significantly more frequent than in normals for CD (19 of 43, $p < 0.01$) and for UC (12 of 25, $p < 0.01$), and absolute values were also significantly higher than in normals for CD (median <39, range <39-1435 nkat/L, $p < 0.001$) and for UC patients (median <39, range <39-1550 nkat/L, $p < 0.001$). As shown in Figure 13.2, only 3 of 63 patients with miscellaneous non-colonic disease and 5 of 41 patients with miscellaneous colonic disease had detectable GE. Neither these frequencies nor the absolute values differed significantly from those for the normal group.

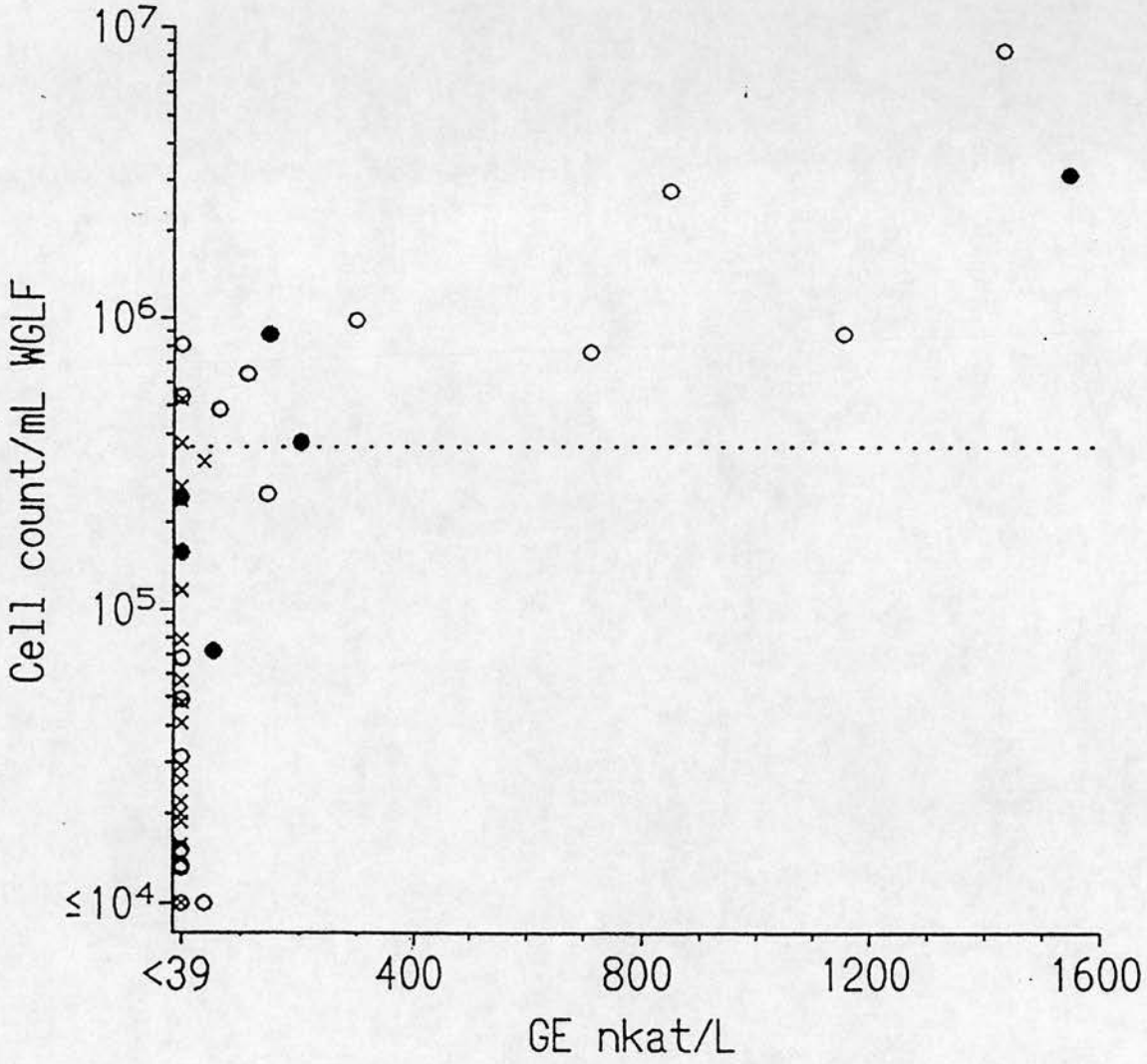

Figure 13.1. Cell count/mL WGLF plotted against GE concentration in WGLF. o = CD, • = UC, x = all other diagnosis. ····· = shows the count/mL equal to the mean + 2SD of the normal group.

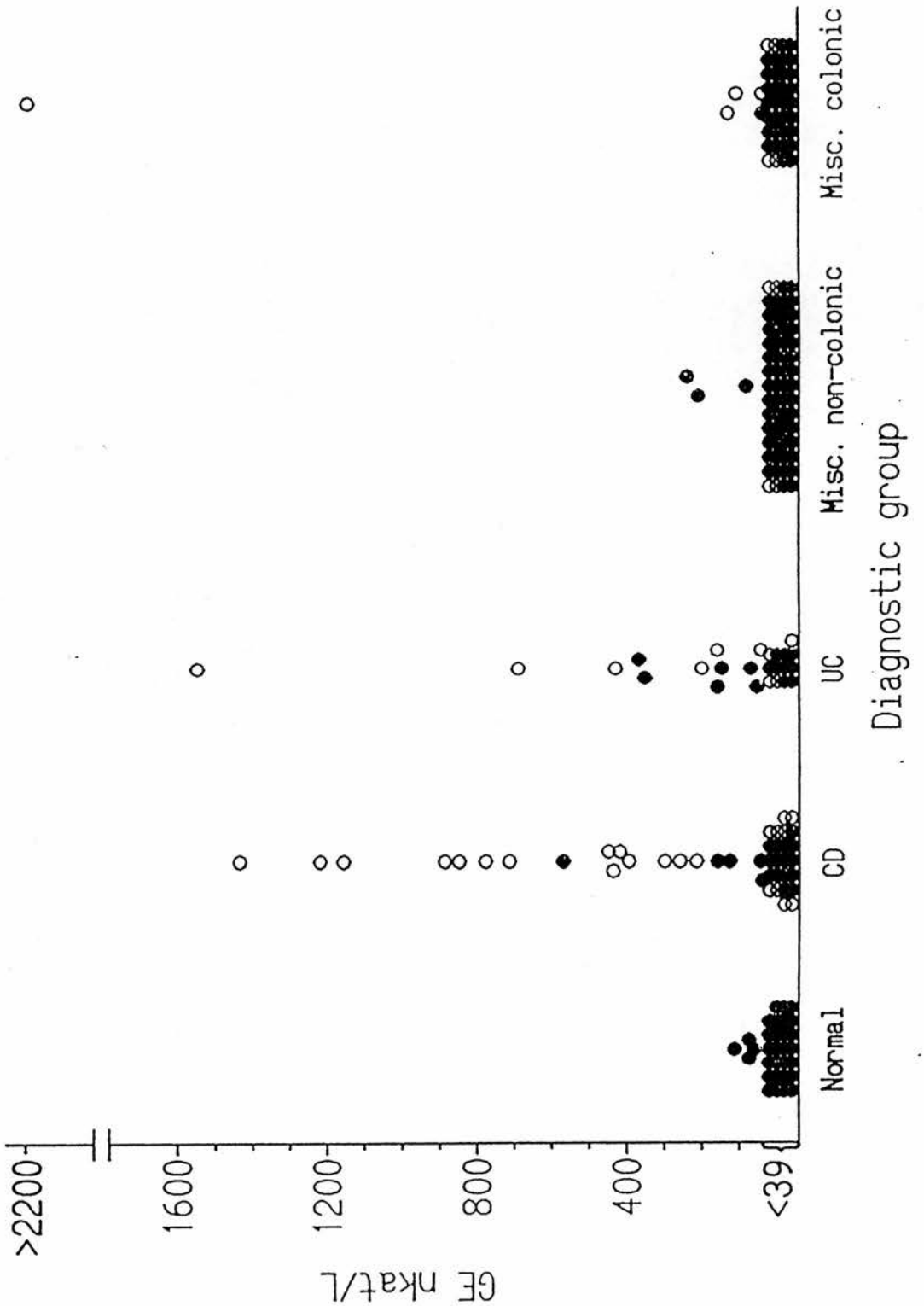

Figure 13.2. Concentration of GE in WGLF plotted against diagnostic group, showing patients with high WGLF IgG concentrations as a measure of active disease. o = WGLF IgG > 10 μg/mL, • = WGLF ≤ 10 μg/mL.

13.3.5. GE concentration in IBD according to disease activity and anatomy of involvement

As summarised in Table 13.2, both the frequency of detection and absolute values for GE concentrations were significantly higher than in normals for the active CD and active UC patients, but not for inactive disease. Active CD specimens also had significantly higher GE levels than inactive CD ($p < 0.01$), but the difference between active and inactive UC failed to reach statistical significance.

When the frequency of detection and absolute values for GE were examined in active CD patients after subdivision by distribution, differences

Table 13.2. Detection of GE in IBD patients and normals

Diagnostic group	n	GE present	p Vs Normals	p Vs other relevant groups	WGLF GE conc (nkat/L) median (range)	p Vs Normals	p Vs other relevant groups
Normal	31	4			<39 (<39-113)		
Active CD	23	14	<0.01		300 (<39-1435)	<0.001	
Inactive CD	20	5	NS	<0.05 vs active CD	<39 (<39-571)	NS	<0.005 Vs active CD
Active CD:							
Colonic	11	10	<0.02		437 (<39-1435)	NS	
Non-colonic	12	4	NS	0.02 Vs colonic CD	<39 (<39-887)	NS	<0.02 Vs colonic CD
Active UC	10	6	<0.01		103 (<39-1550)	<0.001	
Inactive UC	15	6	NS	NS Vs active UC	<39 (<39-370)	NS	NS Vs active UC

emerged between colonic and non-colonic CD, confirming what we had observed by cytology in a smaller series, that an excess of granulocytes in WGLF is a feature mainly of colonic disease. The active colonic CD group contained significantly more samples with detectable GE (10/11) than the active non-colonic CD group (4/12) ($p<0.02$). The GE concentration in active colonic CD was greater than that in active non-colonic CD ($p<0.02$) or, that in small bowel CD alone

($p<0.05$). There was no such differences with inactive disease. No difference in the frequency of GE detection could be shown between patients with pancolitis, distal colitis and proctitis, but the numbers in each of these subgroups were small.

13.3.6. Correlation of WGLF IgG with GE

For the 43 patients with CD, WGLF IgG correlated weakly with GE ($r = 0.36$, $p < 0.02$), with a stronger correlation in UC ($r = 0.58$, $p = 0.002$). For all colonic CD, GE significantly correlated with IgG ($r = 0.44$, $p < 0.01$), but for those with small bowel CD there was no such correlation ($r = 0.01$, p = NS). In all, 43 patients had detectable GE and in these patients GE correlated significantly with WGLF IgG concentration ($r=0.77$; $p<0.0001$).

There were 9 patients with CD and 4 UC patients who had raised IgG concentrations but undetectable GE. More details of these atypical patients are given in Table 13.3. It can be seen that CD patients with raised WGLF IgG concentration but undetectable GE had either small bowel disease or were recently treated with antibiotics. There were no similar common factors in UC patients apart from the fact that they were all on either systemic or local steroids.

13.3.7. Correlation of GE with conventional laboratory indices of activity

For CD patients, GE levels in WGLF were significantly correlated with plasma CRP ($r = 0.49$, $p = 0.001$) but not with ESR, white cell or platelet count in the blood. For the UC group, GE was significantly correlated with ESR ($r = 0.50$, $p < 0.01$) but not with blood white cell or platelet count, or with CRP.

13.3.8. GE concentrations according to treatment status

Table 13.4a shows the GE results for the IBD patients, subdivided according to disease activity and treatment status. When separately stratified by disease activity, patients who were or were not receiving systemic steroid therapy had generally similar frequency of detection and concentrations of GE. In fact, GE concentrations were significantly higher in patients with active CD who were receiving systemic steroids ($p < 0.05$) than in active CD patients not receiving steroids, although the WGLF IgG concentrations were similar for the two groups. Three patients with CD were on azathioprine and they have not been included in

the table. Two of them had inactive disease with undetectable levels of GE; one had active Crohn's colitis with a GE concentration of 450 nkat/L.

Table 13.3. IBD patients with raised IgG (>10 μg/mL) but undetectable GE (<39 nkat/L) in WGLF

Macroscopic distribution	On steroids	IgG (μg/mL)	Comments
Crohn's disease			
Jejunal/ileal	No (TPN)	53	Multiple resection, fistulae
Ileal	Yes	23	On 15 mg prednisolone
Ileal	No	58	
Ileal	No	16	
Ileal/perineal	No	40	Anastomotic recurrence
Ileal	No	142	On tetracycline
Sclerosing cholangitis	No	76	Macroscopically normal but colonic histology showed CD
Colonic	No	182	Had received long course of tetracycline
Perineal	No	13	Solely perineal (had treatment with metronidazole)
Ulcerative colitis			
Pancolitis	Yes	119	On 40mg prednisolone
Pancolitis	Yes	25	On 7.5mg prednisolone
Pancolitis	Yes	217	On 60mg methylprednisolone
Proctitis	Yes	28	On predfoam enema

Note: TPN = Total parenteral nutrition

13.3.9. GE in non-IBD patients

One subject classified as normal, and 5 with diseases other than IBD had high GE concentrations (> 100 nkat/L). These patients are described in Table 13.5. All these 5 patients had clinical disorders compatible with exudation of neutrophils into the lumen. Acute radiation colitis, diverticulitis and pericolic abscess communicating with the lumen are well known to have pus on the mucosa. The other two patients were on NSAIDs. The normal subject was a volunteer in a study investigating mucosal immunity in smokers; a repeat lavage done after 3 weeks did not detect GE in WGLF.

Table 4a. Comparison of total GE concentrations in WGLF from IBD patients on systemic steroids and not on systemic steroids. WGLF IgG concentration is given for reference.

Diagnosis	On systemic steroids				Not on systemic steroids			
	n	No of patients with GE present	GE nkat/L median (range)	IgG μg/mL median (range)	n	No. of patients with GE present	GE nkat/L median (range)	IgGμg/mL
CD								
Active	8	7	576(<39-1435)*	31(16-280)	14	6	<39(<39-1157)*	56(13-216)
Inactive	9	3	<39(<39-127)	2 (1-9)	9	3	<39(<39-571)	4(2-9)
UC								
Active	5	2	<39 (<39-1550)	119(25-244)	5	4	203 (<39-693)	29(13-195)
Inactive	4	3	111(<39-353)	5(2-9)	11	3	<39(<39-370)	2(1-10)

*p<0.05 Vs CD not on steroids

Table 4b. Comparison of free GE concentration in WGLF from IBD patients on steroids and not on steroids.

Diagnosis	On steroids				Not on steroids			
	n	No with free GE	Free GE nkat/L	IgG μg/mL	n	No. with free GE	Free GE nkat/L	IgGμg/mL
CD	9	6*	67 (<39-206)*	29 (4-89)	9	1	<39(<39-81)	23 (2-216)
UC	5	2	<39 (<39-139)	7(6-244)	6	2	<39 (<39-58)	21(5-195)

*p<0.05 Vs CD not on steroids

Table 13.5. Non-IBD patients with markedly raised GE (>100 nkat/L) in WGLF

Diagnosis	IgG μg/mL	GE nkat/L	Comments
Acute radiation colitis	236	2285	Radiotherapy for carcinoma cervix
Diverticulitis	27	132	Settled with antibiotics
Diverticular disease	3	240	On NSAIDs
Carcinoma colon	44	109	Caecal tumour with pericolic abscess
Systemic lupus erythematosus	1	210	On NSAID & prednisolone 10 mg/day
Volunteer	1	113	Normal volunteer; repeat lavage after 3 weeks did not detect GE.

13.3.10. Total and free GE

Aliquots of WGLF which had been filtered, but not treated in any other way, were available for 39 of the 43 specimens in which GE had been detected in unfiltered specimens. Results of GE assays in these paired samples are summarised in Table 13.6. Figure 13.3 shows the relationship between total (particulate+free) and free GE. There was a significant correlation between free GE and total GE ($r=0.8$; $p<0.0001$). Eleven out of the 29 IBD patients with detectable total GE had detectable free GE, whereas none of the 3 normals and only 1 out of the 7 diseased controls (the patient with radiation colitis) had detectable free GE. In the whole group, the concentration of total GE in those with detectable free GE (median 703nkat/L;range 55-2285nkat/L) was significantly greater ($p<0.001$) than that in those with undetectable free GE (median 159nkat/L; range 39-1157nkat/L). This was also true for CD patients in whom the concentration of total GE in those with detectable free GE (median 714nkat/L; range 300-1435nkat/L) was significantly greater ($p<0.05$) than that in those with undetectable free GE (median 215nkat/L; range 39-1157nkat/L). In UC patients, the difference in total GE concentration between those with detectable free GE (median 532nkat/L;range 55-1550nkat/L) and those with undetectable free GE (median 162nkat/L;range 44-432nkat/L) did not reach statistical significance.

In a series of 18 CD patients, disease activity was comparable in the 7 who had free GE (median WGLF IgG 32μg/ml;range 18-89μg/ml) and 11 with no free GE (median WGLF IgG 9; range 2-216μg/ml; p=NS). Five of these patients had colonic disease, 1 had ileal disease (also had ankylosing spondylitis, was on

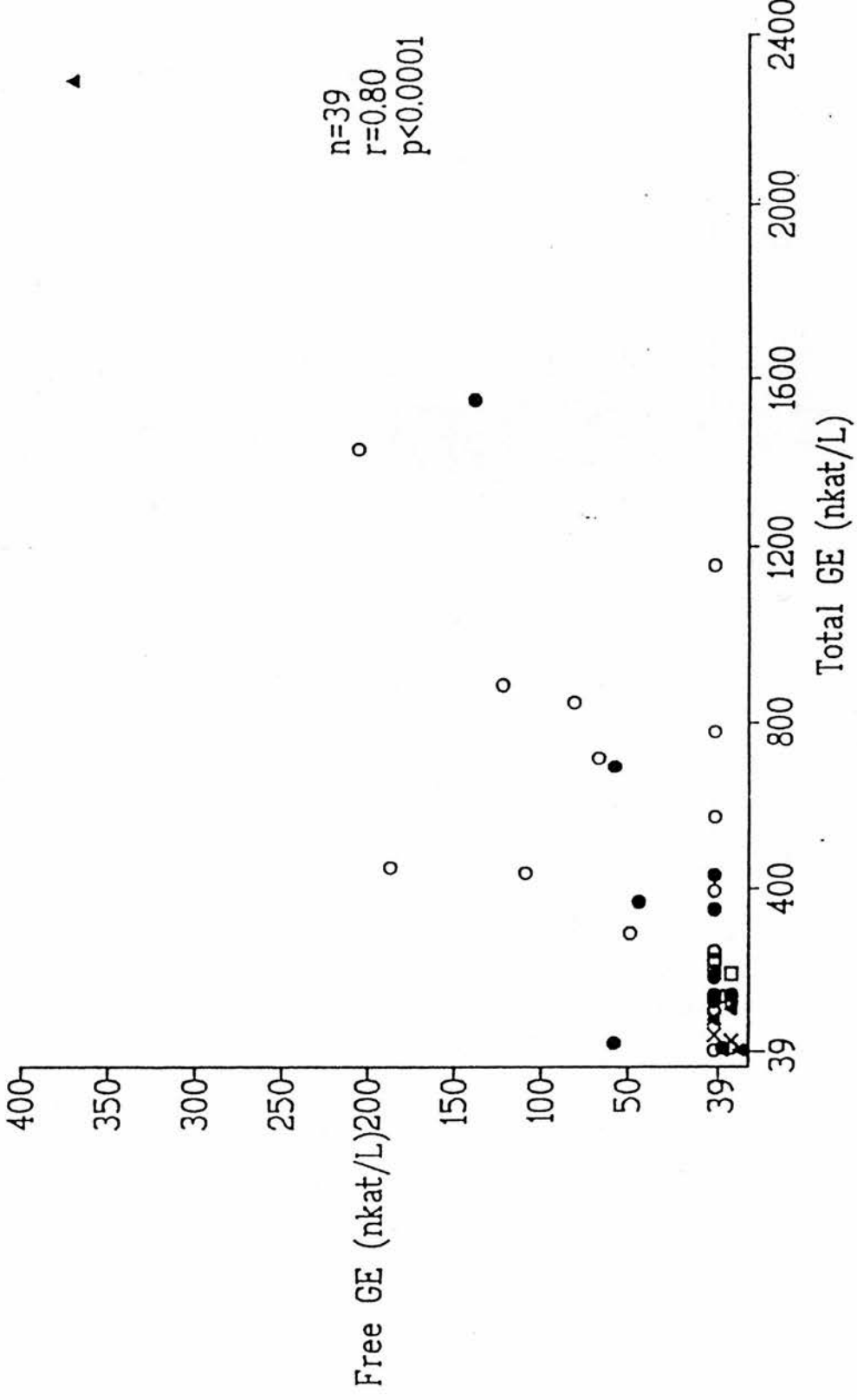

Figure 13.3. The relationship between free and total GE in 39 patients with detectable total GE. x = Normals (n=3); o = CD (n=18); o = UC (n=11); □ = Miscell. upper GI (n=2); Δ = Miscell. colonic (n=5).

Table 13.6. Detection of free GE in 39 patients with detectable total GE.

Diagnostic groups	No. With detectable total GE	No. With detectable free GE
Normals	3	0
CD	18	7
UC	11	4
Miscell upper GI	2	0
Miscell colonic	5	1*

*radiation colitis

NSAIDs and had abscess communicating with ileum), and 1 had perianal disease with rectal involvement. Just as was the case with total GE, free GE was detectable more often in CD patients on steroid therapy (table 4b). Five out of 8 CD patients on steroids and 1 on azathioprine had detectable free GE whereas only 1 out of 9 CD patient not on steroids had detectable free GE ($p<0.05$). The WGLF IgG concentration between this steroid (or azathioprine) treated and untreated group did not differ significantly (median 29(4-89) Vs 23(2-216)μg/ml).

Out of the 11 UC patients with detectable total GE, 4 had free GE. WGLF median IgG concentration in 4 UC patients with detectable free GE was 26μg/ml(range 5-244μg/ml) and in 7 UC patients with undetectable free GE was 9μg/ml (range 6-195μg/ml; p=NS). Two out of 4 UC patients with free GE detectable were on steroids and WGLF IgG concentrations in UC patients on steroids was not different from UC patients not on steroids.

13.4. Conclusion

GE in WGLF is a good surrogate marker of neutrophils in WGLF. Since the method may readily be combined with preparation for radiological or colonoscopic investigation of the bowel, material from patients with gut lesions other than IBD may readily be studied to determine the specificity of particular immunological parameter for a defined pathology. Regulatory mechanisms underlying anatomical differences between small and large bowel diseases may be studied, the effects of pharmacological agents such as steroids, antibiotics and NSAIDS may also be investigated and therapeutic endpoints such as remission of inflammatory activity can be objectively defined.

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Chapter XIV

INVESTIGATION OF PROINFLAMMATORY CYTOKINES IN GUT BY ANALYSIS OF WHOLE GUT LAVAGE FLUID

14.1. Introduction

Cytokines and their roles in IBD are discussed in chapter II. A major difficulty in studying the role of cytokines in relation to inflammatory and metabolic aspects of IBD is that serum levels of cytokines are unlikely to reflect events at the gut mucosal level. Until now, it has therefore been necessary to study tissues (biopsies) by a variety of methods, immunologic, immunohistochemical or molecular (Brynskov *et al* 1992a, 1992b). Tissues from stomach, duodenum and colon from symptomatic patients are relatively easily obtained at endoscopic procedures. However, the small bowel mucosa is difficult to study and data from resection specimens must be interpreted with great caution. Endoscopic biopsy of the large bowel is invasive and serial studies to investigate changes with therapy are difficult to conduct. Healthy controls are almost impossible to recruit for invasive studies. My predecessor (Mwantembe, PhD thesis, University of Edinburgh, 1992) had established that cytokines such as TNF-α could be detected in whole gut lavage fluid (WGLF). I aimed to extend his study to investigate the potential of WGLF analysis for investigating proinflammatory cytokines (IL-1β, IL-8, IL-6, IFN-γ) thought to be involved in gut inflammation.

14.2. Subjects and methods

Filtered and processed WGLF from 234 patients were analysed for IL-1β and IL-8. The methods of assaying for these cytokines are described in chapter XI. All specimens were also assayed for IgG and granulocyte elastase (GE) as described in chapter XI. Table 14.1 describes the patient characteristics. Both a normal control group and two groups of diseased controls, those with upper GI and small bowel diseases and those with colonic diseases other than IBD were studied along with IBD patients. Some were the same patients described in chapters XII and XIII.

Table 14.1. Patient characteristics

Patient group	n	Age median (range)	Sex (M:F)
Normal GI tract	25	43 (22 - 67)	14:11
Crohn's disease			
Active	51	34 (14-62)	30:21
Inactive	29	31 (16-59)	18:11
Ulcerative colitis			
Active	17	30 (18-60)	8:9
Inactive	15	34 (22-58)	9:6
Other non-colonic	57	46 (20-76)	30:27
Other colonic	40	48 (19-82)	22:18

The patient cohort was slightly different for IL-6 and IFN-γ assays. 226 filtered and processed WGLF samples were analysed for IL-6 and IFN-γ. These included 42 patients with normal GI tract, 48 patients with Crohn's disease (CD) (24 active disease with WGLF IgG > 10μg/mL; 24 inactive disease with WGLF ≤ 10μg/mL), 22 patients with ulcerative colitis (UC) (7 active; 15 inactive), 66 patients with miscellaneous noncolonic GI diseases and 48 patients with miscellaneous colonic diseases.

The methods of analyses of cytokines are detailed in chapter XI. Commercial ELISA kits were used for assaying IL-1β and IL-8, while IL-6 and IFN-γ were measured using in-house assays developed by Mrs Louise handy. Statistical analysis was performed using the Mann-Whitney U test and Spearman rank correlation test.

14.3. Results

IL-1β, IL-8, IFN-γ and IL-6 could be detected in WGLF from some patients. In patients with IBD, the results were analysed according to the activity of disease determined by WGLF IgG concentration as described in previous chapters. Granulocyte elastase (GE) concentration was used as a surrogate marker of luminal migration of neutrophils as described in the previous chapter.

14.3.1. WGLF IL-1β concentration

Figure 14.1 shows WGLF IL-1β concentration in the various diagnostic groups. IL-1β was detectable in low concentrations (6.8, 8.6 and 14.4 pg/mL) in 3 patients with normal GI tract and in the remaining 22 with normal GI tract, it was undetectable. One of the three with detectable IL-1β was a healthy volunteer for research on intestinal immunity in smokers. One patient had a normal GI tract but had presented with weight loss. The remaining patient with detectable IL-1β and normal GI tract had a history of alcohol abuse and depression but a normal GI tract. Both these patients also had detectable GE in WGLF and admitted to occasional analgesic use.

In the 51 patients with active (as defined in chapter XII) CD, WGLF IL-1β concentration was significantly greater than the normals (table 14.2). All but one patient with active CD had detectable IL-1β (table 14.3). The exception was a woman with colonic CD and pyoderma gangrenosum who was on metronidazole and salazopyrin. Interestingly, granulocyte elastase too, was undetectable in her WGLF. The concentration of WGLF IL-1β in active CD was significantly greater than in inactive CD (table 14.2). It can be noted that out of the 29 inactive CD patients, 14 had detectable IL-1β and the median concentration of WGLF IL-1β was significantly greater than that in the normals.

All 17 patients with active UC had detectable IL-1β in WGLF and IL-1β concentration was significantly greater than that in the normals (table 14.2). Ten out of the 15 patients with inactive UC also had detectable IL-1β (table 14.3) and the median concentration was significantly greater than that in the normals but significantly less than that in active disease. There was no significant difference in WGLF IL-1β concentration between UC and CD, either for active or for inactive disease.

Twenty-one out of the 57 patients with a variety of non-colonic diseases had detectable IL-1β in WGLF, but the concentrations were low (maximum 71.6 pg/mL) and the median concentration was not significantly different from that in the normals. Three of these patients were on nonsteroidal anti-inflammatory drugs, 3 patients were investigated for iron-deficiency anaemia (one had gynaecological cause for her anaemia, one had extensive colonic diverticulosis and in one, the

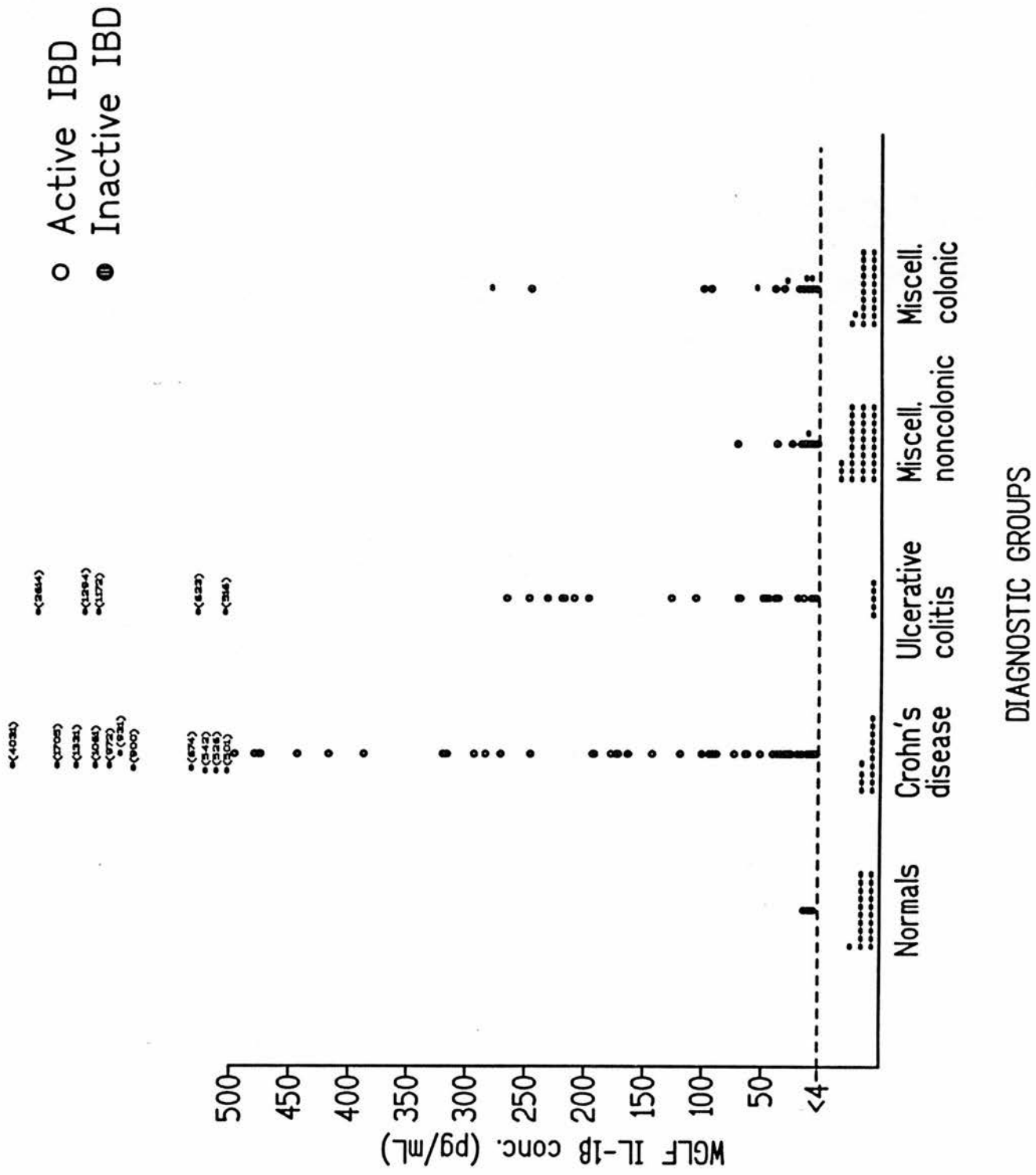

Figure 14.1. WGLF IL-1β concentration in the different diagnostic groups.

diagnosis remained unresolved), 3 patients had bacterial colonisation of the small bowel, and 1 patient each had mesenteric ischaemia, diarrhoea following renal transplant, autonomic neuropathy, common variable immunodeficiency, chronic pancreatitis, giardiasis, bile acid malabsorption, diarrhoea in a patient with lymphoma in remission, short bowel syndrome following resection for malrotation and intestinal atresia in infancy, and diarrhoea in a patient with multiple problems including previous radiotherapy and IgA deficiency. The remaining two patients had transient idiopathic diarrhoea which resolved within a month of the whole gut lavage.

Eighteen out of 40 patients with a variety of colonic diseases had detectable IL-1β in WGLF (table 14.4). Four of these patients had a concentration greater than 100pg/mL as shown in table 14.5. Of the remaining patients with detectable IL-1β, 5 had carcinoma of the colon, 3 had radiation colitis, 2 had infective colitis, and one each had ischaemic colitis, bleeding colonic polyp, tenesmus with possible proctitis and anal fissure (possible Crohn's). The median concentration of IL-1β in this other colonic diseases group was significantly greater than normal but less than active CD or UC.

14.3.2. WGLF IL-8 concentration

This is shown in figure 14.2 and table 14.6. None of the 25 patients with normal GI tract had detectable IL-8 in WGLF (table 14.7). Twenty-three out of the 51 patients with active CD had detectable IL-8 (table 14.7) and the median IL-8 concentration was significantly higher than that in normals (table 14.6). Only one patient with inactive CD had detectable IL-8 in WGLF. This patient with very complicated juvenile CD, multiple surgery and an intra-abdominal abscess had a WGLF IgG concentration of 8μg/mL (upper limit of normal=10μg/mL) and a GE concentration of 65 nkat/L. His IL-1β concentration was 163.8pg/mL.

Table 14.2. Whole gut lavage fluid IL-1β concentration in different diagnostic groups.

Diagnostic groups	n	Median (range) pg/mL	Mean (SD)pg/mL	p vs Normals	p Vs other relevant groups
Normals	25	<4 (<4-14.4)	<4 (3.9)		
Crohn's disease					
Active	51	193 (<4-4031)	386 (629.8)	<0.00001	
Inactive	29	6.6 (<4-931)	51.8 (173)	<0.002	<0.00001 Vs active CD
Ulcerative colitis					
Active	17	127 (5-2614)	372 (650)	<0.00001	
Inactive	15	20.6 (<4-232)	46.2 (71.3)	<0.0005	<0.005 Vs active UC
Miscell. noncolonic	57	<4 (<4-71.6)	6.2 (11.5)	NS	
Miscell. colonic	40	<4 (<4-1294)	57.8 (209.3)	<0.005	<0.00001 Vs active CD/UC NS Vs inactive CD/UC

Table 14.3. Detection of IL-1β in WGLF according to disease activity in IBD.

	WGLF IL-1β undetectable	WGLF IL-1β detectable
Crohn's disease		
WGLF IgG ≤ 10μg/mL	15	14
WGLF IgG> 10μg/mL	1	50
Ulcerative colitis		
WGLF IgG≤ 10μg/mL	5	10
WGLF IgG> 10μg/mL	0	17

Table 14.4. Detection of IL-1β in WGLF in non-IBD patients

Diagnostic groups	IL-1β undetectable	Il-1β detectable
Normals		
WGLF IgG≤ 10μg/mL	22	3
WGLF IgG>10μg/mL	0	0
Miscell. non-colonic		
WGLF IgG≤ 10μg/mL	36	20
WGLF IgG>10μg/mL	0	1
Miscell. colonic		
WGLF IgG≤ 10μg/mL	21	12
WGLF IgG>10μg/mL	1	6

Table 14.5. Non-IBD patients with WGLF IL-1β conc. > 100pg/mL

Diagnosis	Number of patients (pg/mL)
Carcinoma colon complicating UC	1 (246.4)
Radiation colitis	2 (280.2, 100.8)
Carcinoma colon	1 (1294.0)

Table 14.6. Whole gut lavage fluid IL-8 concentration in different diagnostic groups

Diagnostic groups	n	Median (range) pg/mL	Mean (SD) pg/mL	p Vs normals	p Vs other relevant groups
Normals	25	<18 (<18)	<18 (1.2)		
Crohn's disease					
Active	51	<18 (<18-310)	37.5 (61.5)	<0.00001	
Inactive	29	<18 (<18-108)	<18 (20.0)	NS	<0.00001 Vs active CD
Ulcerative colitis					
Active	17	20 (<18-177.9)	48.7 (55.8)	<0.00001	
Inactive	15	<18 (<18-27)	<18 (7.9)	NS	<0.01 Vs active UC
Miscell noncolonic	57	<18 (<18-56.2)	<18 (8.3)	NS	
Miscell colonic	40	<18 (<18-194)	<18 (30.8)	=0.0001	<0.00001 Vs active CD/UC NS Vs inactive CD/UC

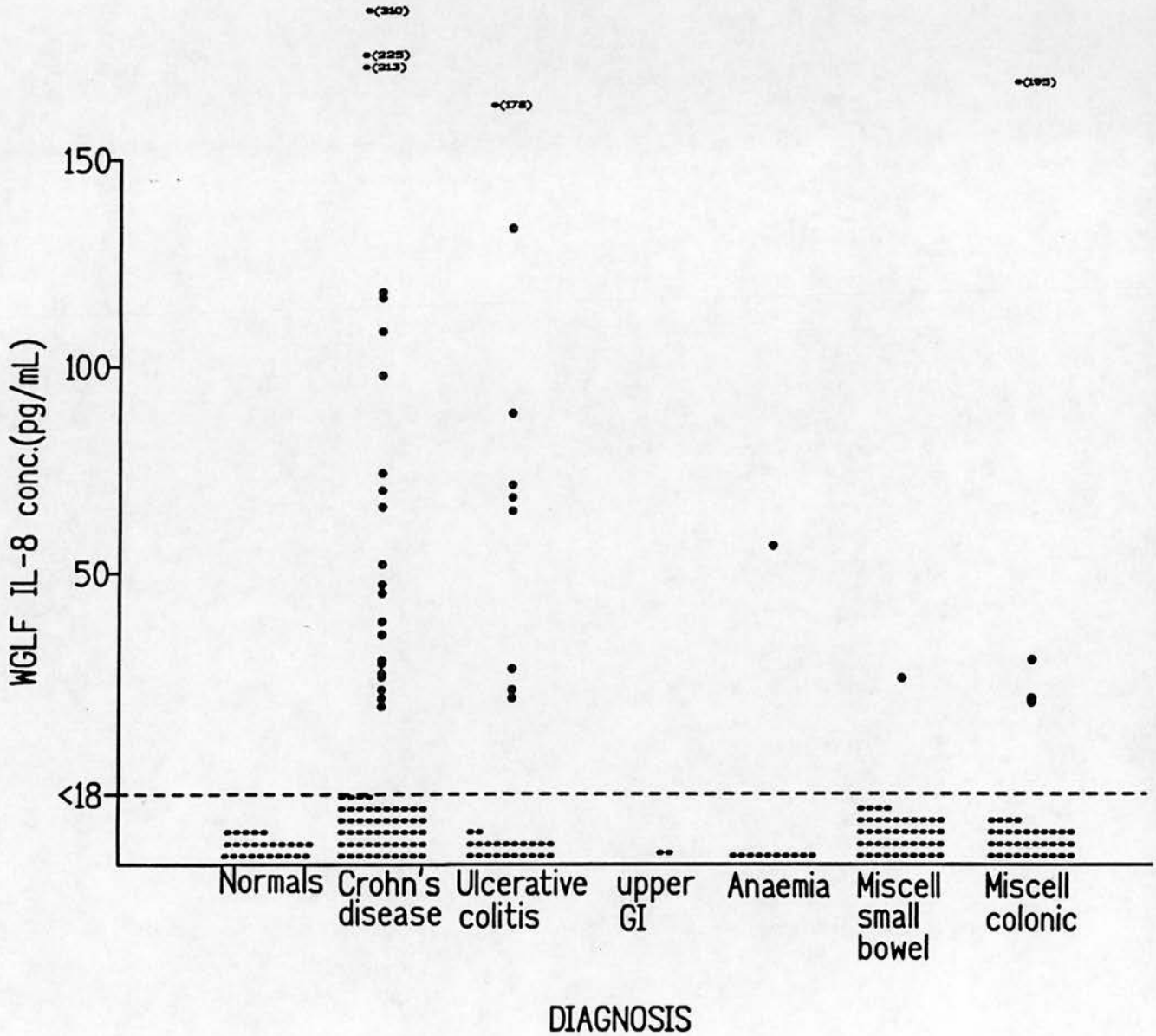

Figure 14.2. WGLF IL-8 concentrations in the different diagnostic groups.

Nine out of 17 patients with active UC had detectable IL-8 in WGLF (table 14.7) and the median IL-8 concentration was significantly higher than that in the normals (table 14.6). Only 1 patient with inactive UC had elevated IL-8 concentration; her WGLF IgG concentration was 7μg/mL, GE concentration was 370nkat/L and IL-1β concentration was 232pg/mL.

Unlike IL-1β, only 8 out of the 97 diseased control patients had detectable IL-8 in whole gut lavage fluid (table 14.8). The details of this group are provided in table 14.9. Most of these patients had low concentrations of IL-8 except one patient with cirrhosis and jejunal varix.

Table 14.7. Detection of IL-8 in WGLF according to disease activity in IBD

	WGLF IL-8 undetectable	WGLF IL-8 detectable
Crohn's disease		
WGLF IgG≤ 10μg/mL	28	1
WGLF IgG>10μg/mL	28	23
Ulcerative colitis		
WGLF IgG≤ 10μg/mL	14	1
WGLF IgG>10μg/mL	8	9

Table 14.8. Detection of IL-8 in WGLF in non-IBD patients

Diagnostic groups	IL-8 undetectable	IL-8 detectable
Normals		
WGLF IgG≤ 10μg/mL	25	0
WGLF IgG>10μg/mL	0	0
Miscell. non-colonic		
WGLF IgG≤ 10μg/mL	1	2
WGLF IgG>10μg/mL	54	0
Miscell. colonic		
WGLF IgG≤ 10μg/mL	30	3
WGLF IgG>10μg/mL	4	3

Table 14.9. Non-IBD patients with detectable WGLF IL-8.

Diagnosis	Number of patients (pg/mL)
Carcinoma of the colon	2 (18.9,19.5)
Jejunal varix, cirrhosis	1 (56.2)
Idiopathic diarrhoea	1 (24.7)
Microscopic colitis	1 (20.0)
Carcinoma colon complicating UC	1 (29.0)
Crippling tenesmus, no diagnosis reached	1 (19.6)
Radiation colitis	1 (194.8)

14.3.3. WGLF IL-1β and IL-8 concentrations in Crohn's disease according to anatomy of involvement

Table 14.10 shows WGLF IL-1β and IL-8 concentrations according to anatomy of involvement after classification by disease activity. For a given disease activity, there was no difference between colonic and ileal involvement for both IL-1β and IL-8. For all anatomical regions of involvement, the concentrations of both cytokines were significantly higher in active disease compared with inactive disease.

Table 14.10. WGLF IL-1β and IL-8 conc. in Crohn's disease according to anatomy of involvement and disease activity.

Activity and anatomy	IL-1β (pg/mL) median (range)	IL-8 (pg/mL) median (range)
Active disease		
Ileal (n=14)	179 (7-1331)	<18 (<18-213)
Colonic (n=31)	193 (<4-4031)	<18 (<18-225)
Other i.e. perineal (n=5)	476 (35-674)	47.1 (<18-310)
Stoma (n=1)	178.4	116.4
Inactive disease		
Ileal (n=5)	<4 (<4-101.4)	<18(<18)
Colonic (n=16)	5.6 (<4-931)	<18 (<18-108.2)
Others (n=6)	13.9 (<4-64.6)	<18 (<18)
Stoma (n=2)	8.5 (<4-17)	<18 (<18)

NOTE. No significant difference in WGLF IL-1β and IL-8 conc. between diseases affecting different anatomical regions for a given disease activity.

14.3.4. Effect of therapy on WGLF cytokines

The effect of therapy on WGLF IL-1β and IL-8 concentrations is shown in table 14.11. For a given disease activity, there was no difference between those on no therapy and those on systemic steroids or an aminosalicylic acid preparation.

The concentrations in those on azathioprine were also similar though the number of patients were small and most were on additional steroid therapy. Clearly, the concentrations become lower once the disease is rendered inactive with therapy, but as shown above, a considerable proportion of these patients continue to have detectable IL-1β in WGLF.

14.3.5. Correlation between neutrophil migration into the gut lumen and IL-8

Concentration of IL-8 in WGLF probably reflects the amount in the lumen. Neutrophil migration into the gut lumen was assessed by GE assay in WGLF as described in chapter XII. I investigated whether luminal IL-8 was the chemoattractant molecule responsible for luminal migration of neutrophils. A correlation between the presence of neutrophils in the lumen (represented by GE concentration in WGLF) and IL-8 is expected, if IL-8 determines neutrophil migration. Correlation of GE with IL-8 concentrations for UC and CD are shown in figures 14.3 and 14.4. In UC, there was a significant correlation between GE concentration and IL-8 concentration ($r=0.78$; $p<0.0001$). In contrast, in CD, there was no correlation between GE concentration and IL-8 concentration ($r=0.16$; p=NS); this was also true for colonic CD only ($r=0.20$; p=NS).

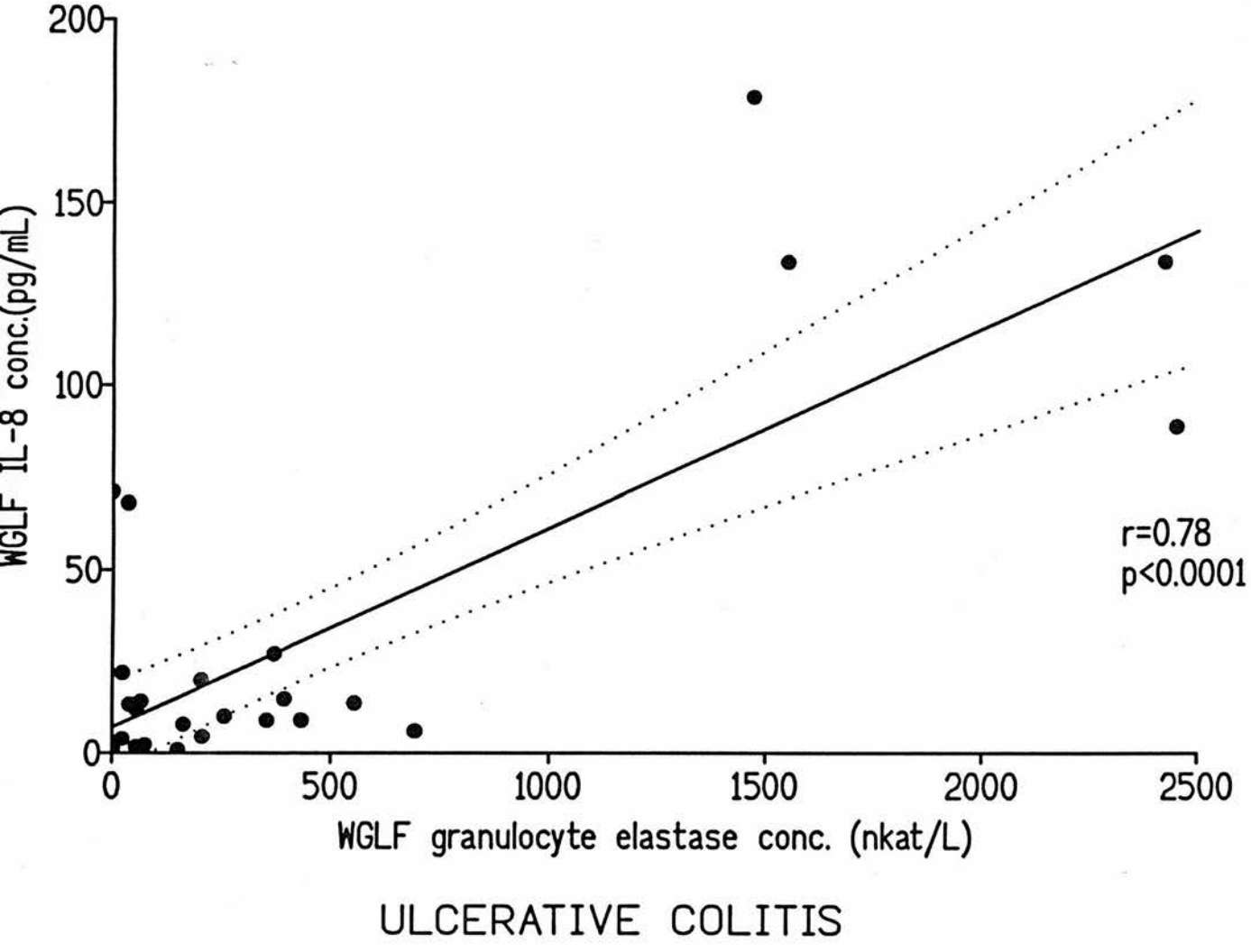

Figure 14.3. Correlation of WGLF GE concentrations with IL-8 concentrations in patients with ulcerative colitis.

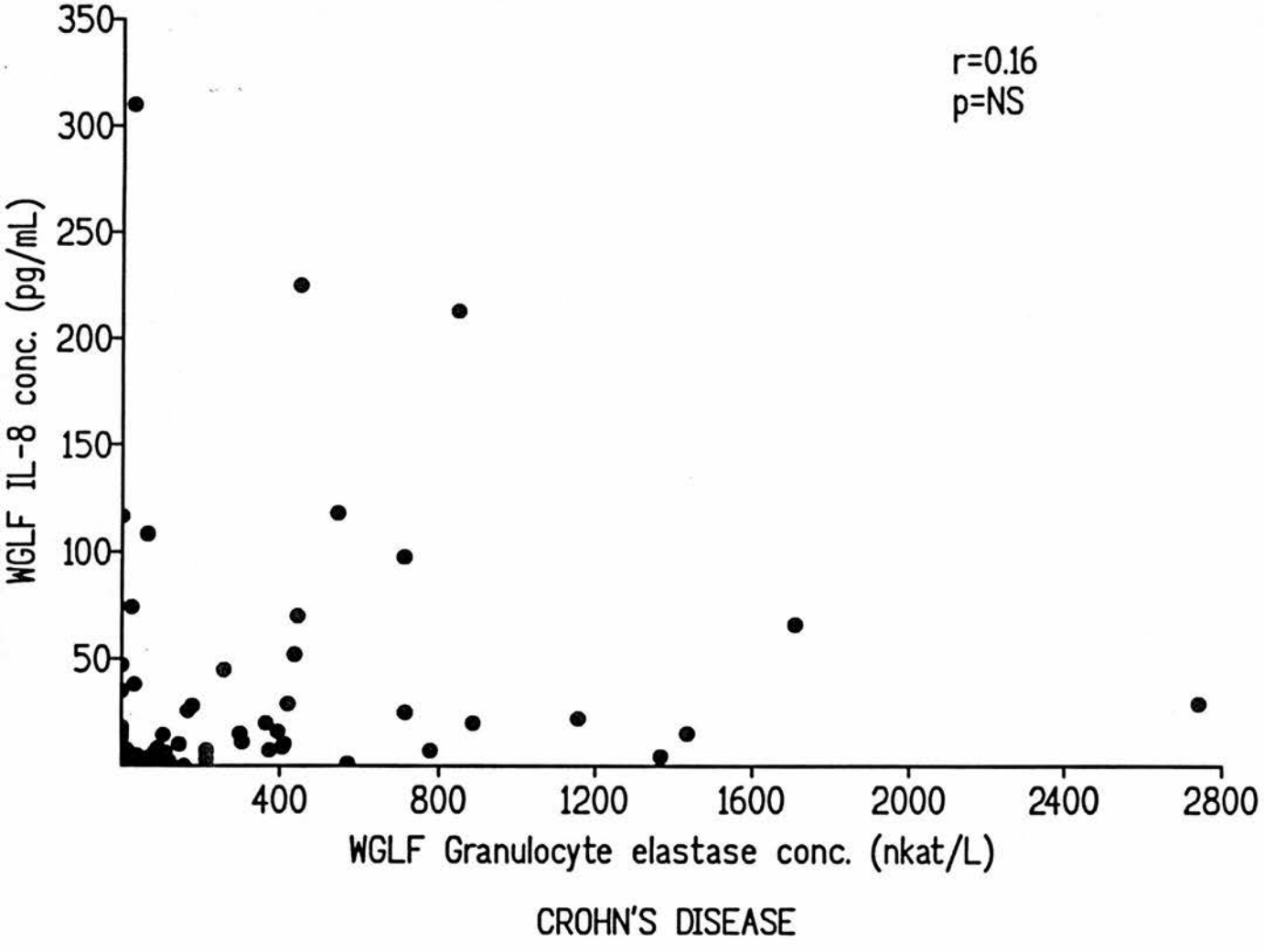

Figure 14.4. Correlation of WGLF GE with IL-8 concentrations in Crohn's disease.

Table 14.11. WGLF IL-1β and IL-8 concentration according to treatment at the time of lavage, for a given disease activity.

Drugs and activity	WGLF IL-1β conc. Median (range)	WGLF IL-8 conc. Median (range)
Active Crohn's disease		
No therapy(n=12)	293.8 (28.4-1061.2)	<18 (<18-213)
Systemic steroids(n==23)	176.2 (<4-1331.2) *(p=NS)*	<18 (<18-116.4) *(p=NS)*
Rectal steroids(n=2)	246.0 (17-474)	34.1 (<18-65.8)
5-Aminosalicylic acid (n=6)	164.4 (10-501) *(p=NS)*	18.0 (<18-45) *(p=NS)*
Steroids + Azathioprine (n=4)	350.0 (27-4031)	22.0 (7.4-38.1)
Azathioprine (n=1)	1705.0	225.0
Steroids + EO28 (n=1)	143.4	<18
EO 28 (n=1)	26.0	18.0
Antibiotics (n=1)	88.8	310.0
Inactive Crohn's disease		
No therapy(n=9)	4.7 (<4-37.8)	<18 (<18)
Systemic steroids(n=15)	8.6 (<4-931) *(p=NS)*	<18 (<18-108.2) *(p=NS)*
Rectal steroids(n=1)	<4	<18
5-aminosalicylic acid(n=3)	10.2 (<4-17.4)	<18
Azathioprine(n=1)	17.0	<18
Active Ulcerative colitis		
No therapy (n=5)	219.8(5.2-266.4)	17.8(<18-64.8)
Systemic steroids(n=8)	322.0(6-2614) *(p=NS)*	79.7(<18-177.9) *(p=NS)*
Rectal steroids(n=2)	43.3(15.8-70.8)	<18 (<18)
5-aminosalicylic acid(n=2)	131.6(45.4-217.8)	<18(<18-20)
Inactive ulcerative colitis		
No therapy(n=7)	23.0(<4-47.6)	<18(<18)
Systemic steroids(n=3)	50 (20.6-197.6)	<18(<18)
Rectal steroids(n=3)	<4(<4-46)	<18(<18)
5-aminosalicylic acid(n=2)	4.5(<4-7)	<18(<18)

All statistical comparisons against patients on no therapy. (EO28=elemental diet)

14.3.6. WGLF interleukin- 6 concentration (Figure 14.5)

Table 14.12 shows the data for detection of IL-6 in WGLF. Four patients with apparently normal GI tract had detectable IL-6. One woman with idiopathic constipation and one man diagnosed as irritable bowel syndrome had other WGLF parameters normal. Of the remaining two, one was a female volunteer in a study on the effect of smoking on intestinal immunity (her WGLF haemoglobin was also raised at 9.6 μg/mL) and another was a patient known to abuse alcohol and suffered from depression (his WGLF GE was modestly raised at 74 nkat/L, IL-1β was detectable at 6.8 pg/mL and WGLF haemoglobin was 9.0 μg/mL).

Table 14.12 Detection of IL-6 in WGLF

Diagnostic groups	IL-6 undetectable	IL-6 detectable	IL-6 conc. median (range) pg/mL
Normal (n=42)	38	4	<20 (<20 - 46.6)
Crohn's disease			
Active (n=24)	19	5	<20 (<20 - 66.8)
Inactive (n=24)	19	5	<20 (<20 - 132.4)
Ulcerative colitis			
Active (n=7)	5	2	<20 (<20 - 35.8)
Inactive (n=15)	12	3	<20 (<20 - 62.6)
Other noncolonic (n=66)	58	8	<20 (<20 - 39.8)
Other colonic (n=48)	43	5	<20 (<20 - 109.4)

Five patients with colonic diseases other than IBD had detectable IL-6. Two of them had acute radiation colitis (one woman had very abnormal WGLF parameters with IgG of 236μg/mL, GE 2285 nkat/L, IL-1β 280.2 pg/mL, IL-8 194.8 pg/mL, α_1-antitrypsin 60 μg/mL, haemoglobin 143 μg/mL; one man had WGLF IgG 29 μg/mL, GE 39 nkat/L, IL-1β 12.6 pg/mL, albumin 78 μg/mL, haemoglobin 5.2 μg/mL). One patient had a carcinoma of the colon (IgG 44 μg/mL, IL-1β 31.8 pg/mL, albumin 115 μg/mL) and one patient had a bleeding colonic polyp (WGLF Haemoglobin 6.3 μg/mL). The remaining patient had a crippling tenesmus for which no satisfactory explanation was found, but he had a low grade atypical proctitis (possibly traumatic) - his IgG was 8 μg/mL, IL-1β was 5.6 pg/mL, IL-8 was 19.6 pg/mL, albumin was 34 μg/mL.

Eight patients with non-colonic diseases other than IBD had detectable IL-6. Three of them had idiopathic diarrhoea, and in two of them the diarrhoea was self-limited (one was antibiotic associated) and had settled at the time of the whole gut lavage (in one IL-1β was detectable and in another WGLF haemoglobin was 32.4 μg/mL, in the remaining patient all other WGLF parameters were normal). One man had diarrhoea with complex features including previous radiotherapy and IgA deficiency (his WGLF IgG was 31 μg/mL, IL-1β 12.4 pg/mL, albumin 29 μg/mL). Four patients were being investigated for iron-deficiency anaemias (one of them had a large hiatus hernia and diverticulitis with WGLF GE of 240 nkat/L, IL-1β of 11.6 pg/mL and haemoglobin of 7.0 μg/mL; one patient had a uterine cause for her anemia, and in the two other patients the cause of anaemia remained unresolved).

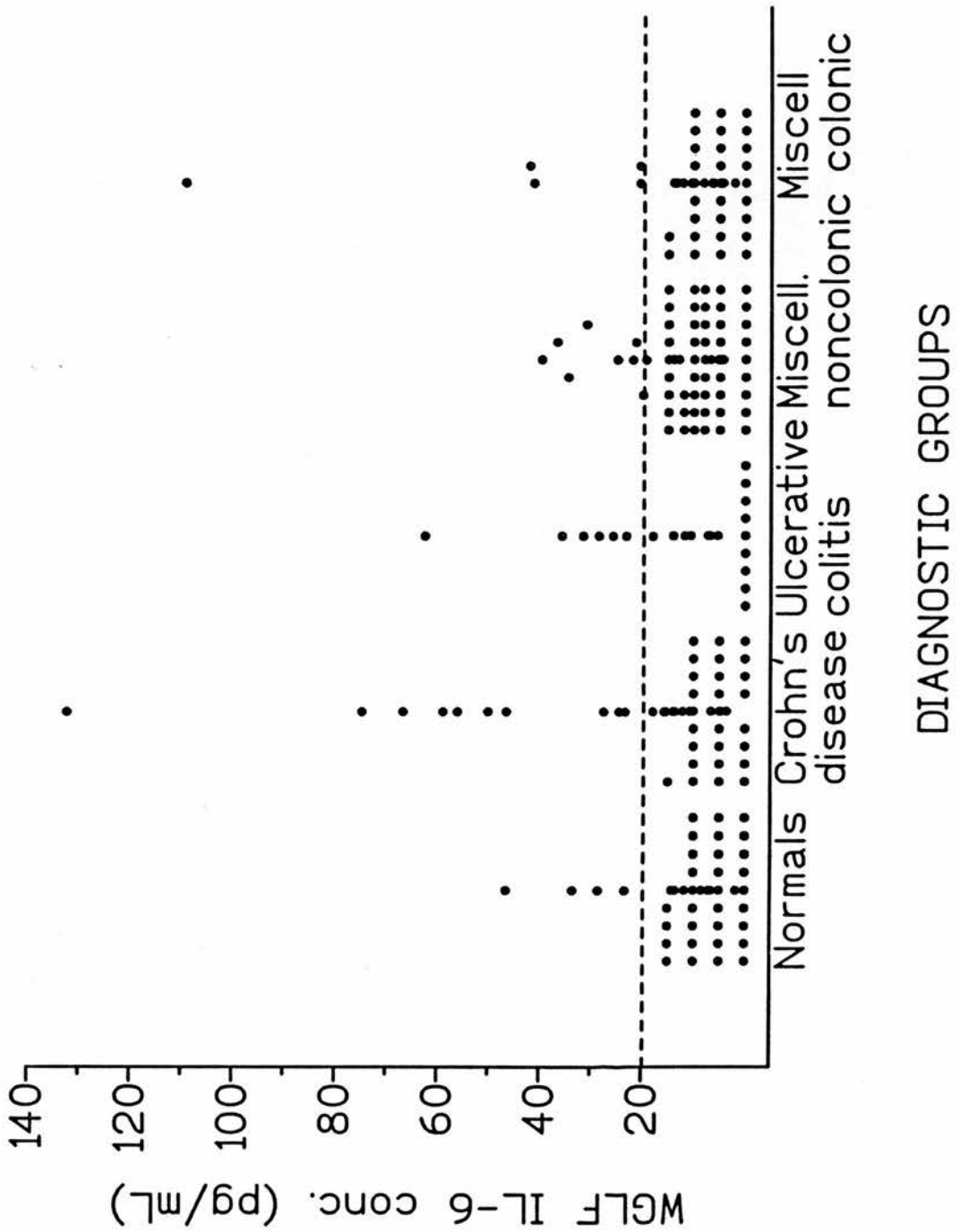

Figure 14.5. WGLF IL-6 concentrations in the different diagnostic groups.

14.3.7. WGLF interferon - γ (IFN-γ) concentration (Figure 14.6)

Detection of interferon - γ in WGLF is shown in table 14.13

Table 14.13. Detection of IFN-γ in WGLF

Diagnostic groups	IFN-γ undetectable	IFN-γ detectable	Median conc. (range) IU/mL
Normal GI tract (n=42)	37	5	<0.64 (<0.64-1.62)
Crohn's disease			
Active (n=24)	19	5	<0.64 (<0.64-4.60)
Inactive (n=24)	20	4	<0.64 (<0.64-3.46)
Ulcerative colitis			
Active (n=7)	3	4	1.78 (<0.64-3.04)
Inactive (n=15)	10	5	<0.64 (<0.64-8.46)
Other noncolonic (n=66)	57	9	<0.64 (<0.64-3.26)
Other colonic (n=48)	42	6	<0.64 (<0.64-3.30)

Five samples derived from patients with apparently normal GI tract had detectable IFN-γ. One of them with a history of alcohol abuse and depression and another (two samples were from her on different occasions) had idiopathic constipation being treated with whole gut lavage - both of them had detectable IL-6 and are described above. Of the remaining patients, one had irritable bowel syndrome (all other WGLF parameters normal) and another had idiopathic constipation (WGLF haemoglobin 5.4 μg/mL).

Six patients with colonic disease other than IBD had detectable IFN-γ. Two of them had ulcerated colonic carcinoma (one also had IL-6 and is described above, the other had WGLF IL-1β of 138.6 pg/mL, albumin 35 μg/mL, α_1-antitrypsin 22 μg/mL and haemoglobin 32 μg/mL). Two patients were post-radiotherapy and were taking part in a study of intestinal immunity after radiotherapy (one of them also had WGLF albumin of 34 μg/mL and haemoglobin of 9.1 μg/mL). One patient had a presumptive diagnosis of infective proctitis which settled (WGLF IgG 33 μg/mL, IL-1 5.6 pg/mL, albumin 84 μg/mL, haemoglobin 22.3 μg/mL). The remaining patient had a colonic polyp and all other lavage parameters were normal.

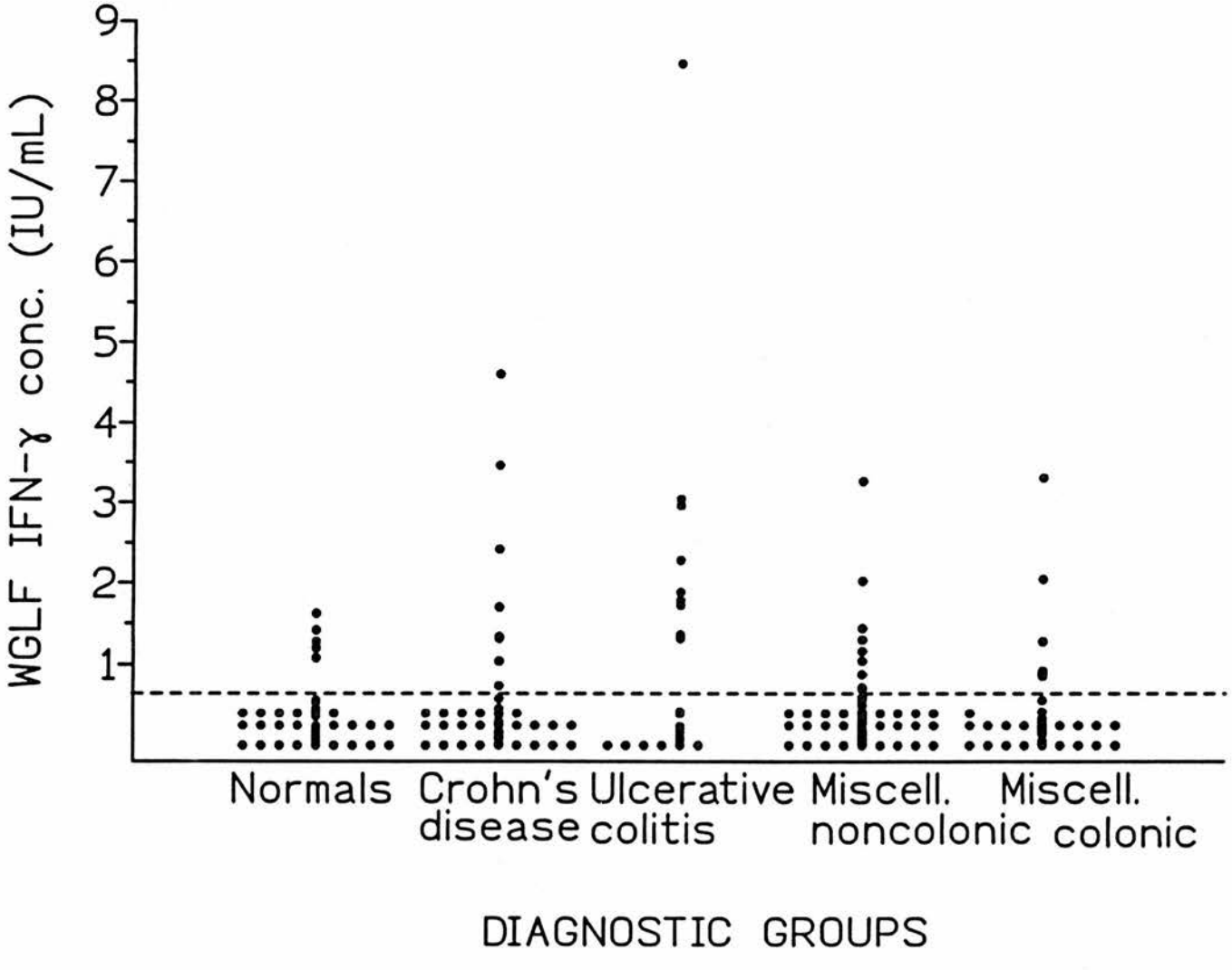

Figure 14.6. WGLF IFN-γ concentrations in the different diagnostic groups.

Nine patients with upper GI or small bowel disease other than IBD (or iron deficiency anaemia being investigated for GI blood loss or coeliac disease) had detectable IFN-γ in WGLF. Five of them had diarrhoea due to a variety of causes (autonomic neuropathy, postgastrectomy, post-renal transplant on immunosuppresives and two idiopathic, sef-limited of whom one had detectable IL-6 described above). Two of them were being investigated for iron-deficiency anaemia (one of them with hiatus hernia and diverticulitis described above and another with inconclusive diagnosis but WGLF IL-1β of 15.8 pg/mL and haemoglobin of 6 μg/mL). One patient had bleeding oesophageal and rectal varices due to cirrhosis and the remaining patient had erosive duodenitis with a small GI bleed.

14.4. Conclusion

WGLF analysis is a noninvasive, convenient method to analyse proinflammatory cytokines produced by an inflamed gut. It is complementary to studies on biopsy specimens and enables large numbers of cases to be studied. Both small and large bowel disease may be investigated. Both WGLF IL-1β and IL-8 concentrations are significantly elevated in active IBD but a considerable proportion of patients with inactive IBD as defined by WGLF IgG concentration (supported by clinical indices of activity and physicians' global assessment) continue to have raised IL-1β concentration. Neutrophil migration into the gut lumen, measured by WGLF GE assay correlates significantly with WGLF IL-8 concentration in UC but not in CD. Other luminal chemoattractants of neutrophils, such as bacterial peptides, may have an important role in luminal migration of neutrophils in CD. Elevated IL-1β and IL-8 in WGLF are not specific for IBD but may be detected in other inflammatory and neoplastic diseases of the gut. IL-6 and IFN-γ are detectable far less frequently in WGLF than IL-1β or IL-8 and usually in association with other abnormal WGLF parameters. The implications of the findings are discussed in chapter XVI.

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Chapter XV

DETECTION OF INSULINLIKE GROWTH FACTOR - 1 AND TRANSFORMING GROWTH FACTOR - β IN WHOLE GUT LAVAGE FLUID: A NOVEL METHOD OF STUDYING INTESTINAL FIBROSIS

15.1. Introduction

Insulinlike growth factor -1 (IGF-1) is a polypeptide of 70 amino acids (7650 Daltons). It is a potent mitogen for fibroblasts and smooth muscle cells and stimulates collagen synthesis. It is secreted by fibroblasts, macrophages and lymphocytes. IGF-1 may be important in development of granulomatous inflammation and fibrosis in a rat model of granulomatous enterocolitis (Zimmermann *et al* 1993). Little is known about its role in human IBD. In plasma, IGF-1 is bound to a family of IGF-1 binding proteins and needs to be released from its bound form before assay.

Transforming growth factor - β (TGF-β), a 25 kilodalton dimer, is a major product of platelet degranulation (Assoian *et al* 1983) and of macrophages (Assoian *et al* 1987), has been identified as one of the mediators that specifically induces fibrotic response in injured tissue (Sporn *et al* 1987). TGF-β increase collagen synthesis by human intestinal smooth muscle cells in cell culture experiments (Graham *et al* 1990). Studies in human IBD are limited and have been reviewed in chapter II.

The aim of the study was to utilize the technique of whole gut lavage to investigate the roles of IGF-1 and TGF-β in inflammatory diseases of the gut characterised by fibrosis.

15.2. Subjects and methods

15.2.1. Subjects

IGF-1 was assayed in 64 filtered, processed WGLF samples. These included samples from 7 patients with normal GI tract, 20 patients with Crohn's disease (CD), 12 patients with ulcerative colitis (UC), 13 patients with current or

previous abdominal radiotherapy and 12 patients with other GI diseases. Seven of the patients in the radiotherapy group were taking part in a study of the effect of radiotherapy on intestinal immunity and inflammation and 6 patients presented to the GI unit with radiotherapy-related intestinal symptoms.

In 56 out of the 64 samples, TGF-β was also assayed. These included 6 patients with normal GI tract, 16 patients with CD, 12 patients with UC, 13 patients with current or previous abdominal irradiation and 9 patients with other GI diseases.

15.2.2. Methods

Both IGF-1 and TGF-β were assayed using commercially available immunoassay kits as described in chapter XI. The lower limit of detection of IGF-1 was 1.1 μg/mL and that of TGF-β was 50 pg/mL. Patients with IBD were considered to have active inflammation if their WGLF IgG concentration exceeded 10μg/mL.

15.3. Results

15.3.1. WGLF Insulinlike Growth Factor - 1

The recovery of IGF-1 from PEG-electrolyte solution spiked with IGF-1 standard was a mean of 92% (range 88%-94%) in five experiments where the concentration of IGF-1 ranged from 10μg/mL to 30μg/mL. IGF-1 could not be detected in WGLF not processed by the addition of protease inhibitors. Figure 15.1 shows WGLF IGF-1 concentrations in the different disease groups. IGF-1 could not be detected in patients with normal GI tract. IGF-1 was detectable in 8 out of 20 patients with CD. One of these patients, a 78-year-old lady with colonic CD and severe diverticular disease with a sigmoid stricture had a very high concentration of IGF-1 in WGLF (158μg/mL). Only 1 out of 12 patients with UC had detectable IGF-1. This patient had chronic active pancolitis for over 12 years only incompletely controlled on cyclosporin and steroids - he was on antipsychotic drugs for schizophrenia and persistently refused surgery. He eventually had a proctocolectomy within a year of having the whole gut lavage. None of the 3 patients with previous or current history of radiotherapy who had detectable IGF-1

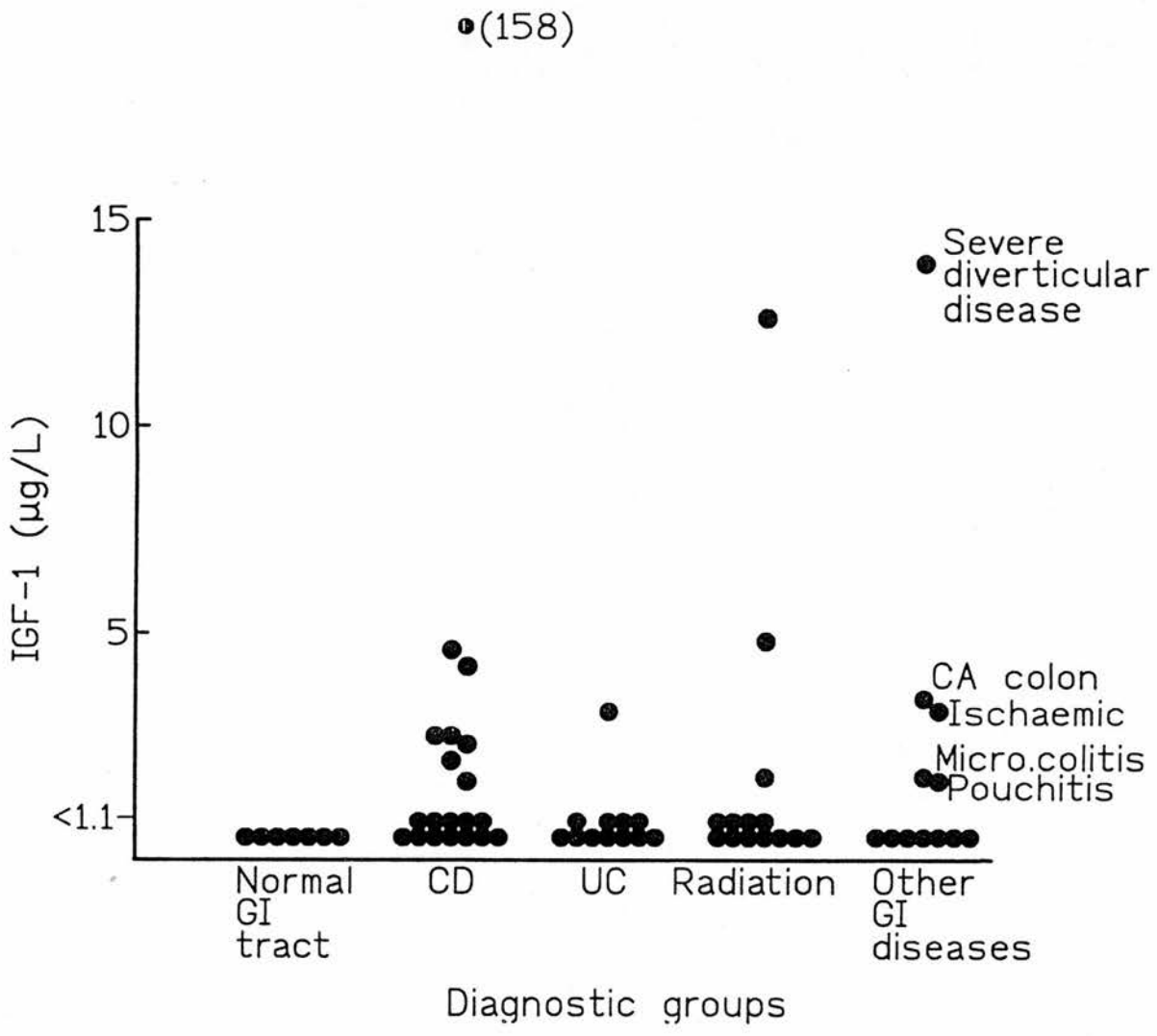

Figure 15.1. WGLF IGF-1 concentrations in different diagnostic groups.

had acute inflammation; WGLF IgG, albumin and α_1-antitrypsin were within the normal range in all three. Two of these three patients were taking part in a study investigating the gut mucosal effects of radiotherapy and the third patient had past radiotherapy induced colonic stricture. Other GI diseases with detectable IGF-1 in WGLF were a patient each with severe diverticular disease with attacks of diverticulitis, microscopic colitis, pouchitis, ischaemic colitis and carcinoma of the colon, all conditions known to be associated with increased fibrogenesis.

Figure 15.2 shows disease activity represented by WGLF IgG plotted against WGLF IGF-1 concentration in CD. There is no correlation between WGLF IgG and IGF-1 concentrations (r=-0.3; p=NS). Table 15.1 shows that IGF-1 was detectable in 6 out of the 16 patients with active CD and 2 out of 4 patients with inactive CD. The frequency of detection of IGF-1 was significantly higher in CD compared with UC ($p < 0.05$).

Table15.1. IGF-1 concentration in IBD according to disease activity.

	IgG conc. μg/mL median(range)	No. of patients with detectable IGF-1	IGF-1 conc.μg/L median(range)
Normal	2 (0-5)	0	<1.1
CD			
Active(n=16)	50 (11-216)	6	<1.1 (<1.1-158)
Inactive(n=4)	1(1-3)	2	<1.1(<1.1-4.2)
UC			
Active(n=10)	38 (13-455)	1	<1.1 (<1.1-3.1)
Inactive(n=2)	5,9	0	<1.1

$p<0.05$ CD Vs UC (χ^2 test with Yates correction).

Four out of 7 patients with ileal or ileocolonic CD and detectable IGF-1 in WGLF had resectional surgery within 3 months of having whole gut lavage and small intestinal strictures were found in all them. One patient with colonic CD had detectable IGF-1 and he had not undergone surgery within 6 months after whole gut lavage. Only one out of the 12 CD patients with undetectable IGF-1 in WGLF had documented radiological strictures.

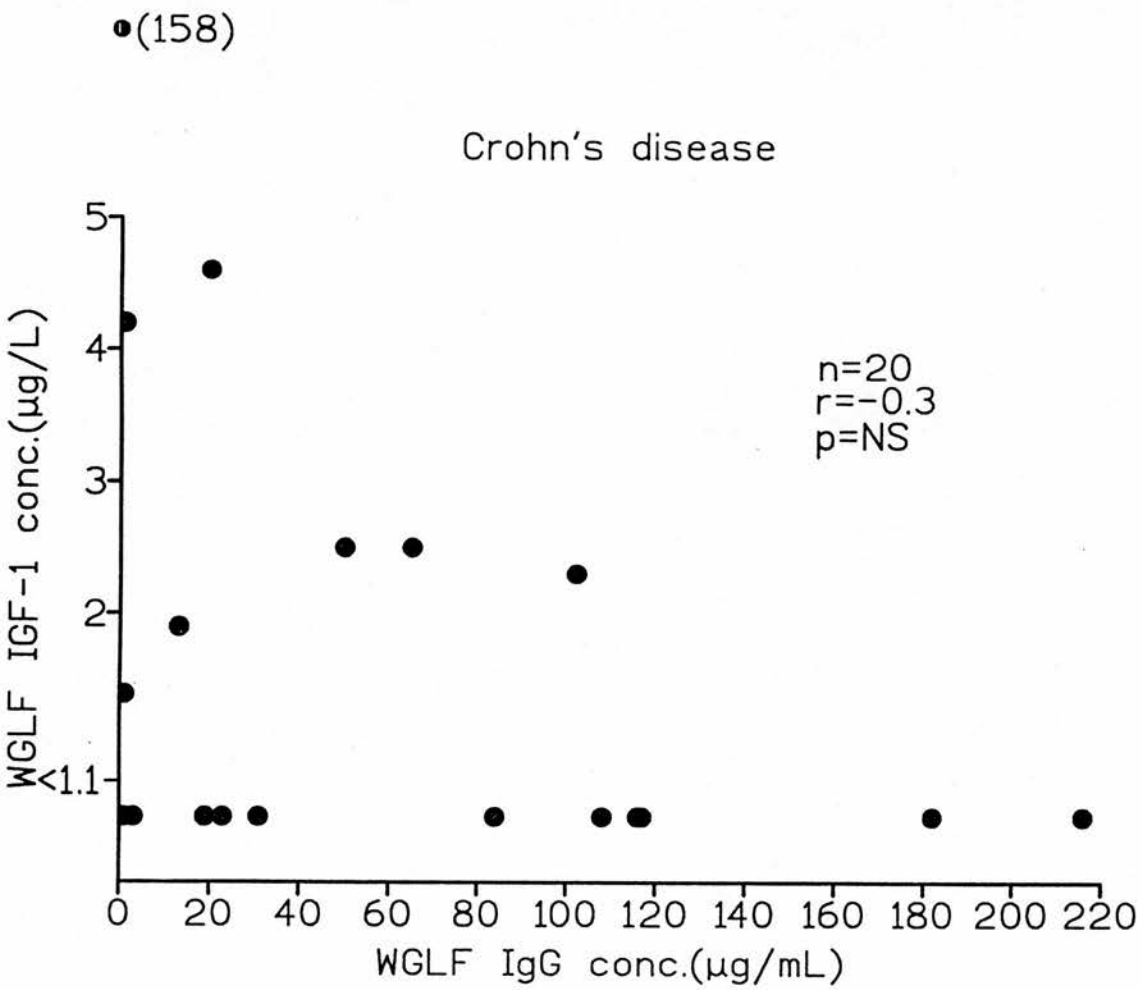

Figure 15.2. WGLF IgG concentrations plotted against IGF-1 concentrations in Crohn's disease.

15.3.2. Transforming Growth Factor - β

The recovery of TGF-β from PEG-electrolyte solution spiked with TGF-β standard was a mean of 96% (92-98%) in five experiments, where the TGF-β concentration ranged from 50pg/mL to 500pg/mL. TGF-β concentration was 10-fold less in WGLF not processed by addition of protease inhibitors compared with processed fluid. The concentration of TGF-β in unfiltered fluid was comparable to filtered fluid. Hence filtered, processed fluid was chosen as an appropriate sample for the assay.

In contrast to IGF-1, TGF-β was detectable in all WGLF samples that were tested. There was no significant difference between those with normal GI tract and any of the different diagnostic groups (Figure 15.3). However, the concentration of TGF-β in patients with current or previous radiotherapy was significantly lower than that in either CD or UC ($p < 0.01$). There was no correlation between WGLF TGF-β and IGF-1 or IgG concentrations.

15.4. Conclusions

The two growth factor peptides, IGF-1 and TGF-β can be detected in WGLF using recently available commercial assays. Detectable WGLF IGF-1 is a feature of CD and other diseases well known to be associated with fibrosis. In contrast, it is infrequently detectable in UC. Disease activity and IGF-1 are not correlated. Detection of IGF-1 in WGLF shows promise as a relatively noninvasive method of studying gut fibrosis in both the small bowel and colon. In contrast, TGF-β was detectable ubiquitously in lavage fluid, but concentrations in patients who had undergone radiotherapy were lower than that in IBD. TGF-β is involved in epithelial repair and the implication of this finding is discussed in the next chapter.

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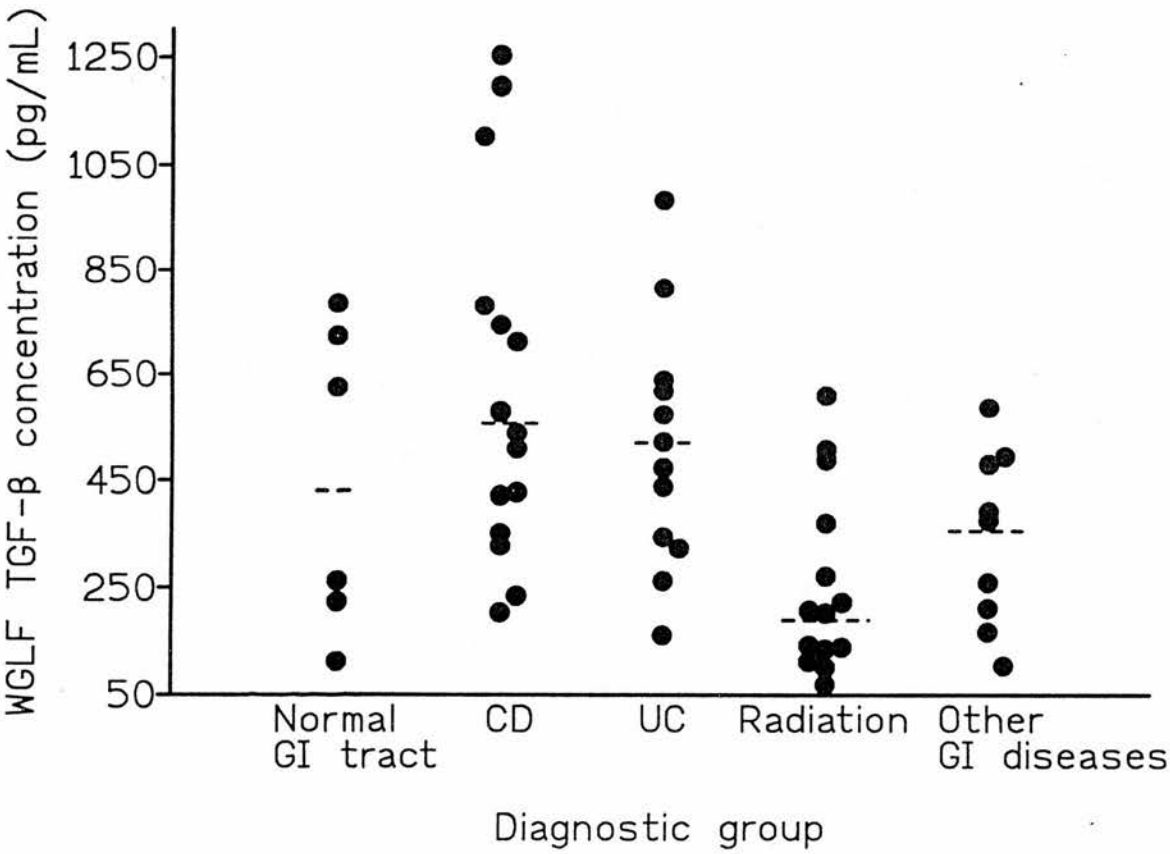

Figure 15.3. WGLF TGF-β concentrations in different diagnostic groups.

Chapter XVI

DISCUSSION: INVESTIGATION OF IBD PATIENTS BY ANALYSIS OF COMPONENTS OF INFLAMMATION IN WHOLE GUT LAVAGE FLUID

There is clearly a need for simple and objective clinical tests to tease apart the different components of inflammation, so that their relevance to different parts of the gut affected, effects of different drug and nutritional therapies and inter-relationship with the gut flora can be evaluated. This work has confirmed that cells of the intestinal lumen can be investigated by peroral whole gut lavage. The influence of the site of macroscopic disease involvement or the effect of drug treatment, such as corticosteroid therapy, on a specific feature, such as luminal neutrophilia, can be examined. Moreover, the finding based on my predecessor's work that the concentration of IgG in whole gut lavage fluid (WGLF) is related to disease activity now allows us to subdivide and stratify IBD patients into groups with comparable disease activity. By applying WGLF analysis to studies of other forms of gastrointestinal disease, it can be determined whether a particular immunological phenomenon is a specific feature of one or both forms of IBD or is simply an expression of a common form of tissue injury in the gut.

When my predecessors studied WGLF concentrations of proteins in IBD patients in relation to "disease activity" (defined on the basis of the widely used CDAI for CD or PTI for UC), highly significant correlations emerged, particularly for IgG (Choudari *et al* 1993). Continuing experience, including the results for patients in this study, has confirmed that WGLF IgG concentration of $\leq$10 μg/mL is measuring the same phenomenon of "inactive disease" as these, mainly subjective, global scores. Such scores are widely used as end points in clinical trials of IBD treatment, and it is possible that objective tests based on WGLF analysis may be valuable as supplementary evidence of efficacy of new treatments. However research on WGLF proteins, together with the work of others using endoscopy (Cellier *et al*), clearly show that "disease activity" is a separate phenomenon from destructive, ulcerative, gross anatomical disease, particularly in CD. Clinical

research methods and laboratory tests to investigate the latter are likely to differ from those which detect disease activity. For example, in a rarely cited but comprehensive study, many patients with "inactive " IBD (on the basis of CDAI<150) in fact had significant gut inflammatory activity as measured by radiolabelled leukocyte excretion (Saverymuttu 1986b).

16.1. Investigation of neutrophil migration into the gut lumen by cytology of whole gut lavage fluid

Many approaches are being used to study gut inflammation and its pathogenesis. These include immunocytochemical investigations of biopsies and resected specimens, radiolabelled leukocyte studies, perfusion of isolated loops of small bowel or colon with measurements of proinflammatory mediators, and various immunological tests on faecal extracts. Radiolabelled leukocyte tests have provided most of the recent descriptive information on the migration of cells into the bowel wall and thence into the gut lumen, but more direct methods are needed to investigate the mediators and chemotactic agents involved.

The use of whole gut lavage for research on intestinal antibodies was described by Gaspari *et al* 1988. Work done by my predecessors and colleagues have simplified and standardised the clinical protocol to create what is essentially a whole gut perfusion system, relatively unaffected by disease, age, drugs or diet (Brian and Ferguson, submitted for publication). After appropriate specimen processing, WGLF analysis can be used to investigate gut immunoglobulins and antibodies (O'Mahony *et al* 1990), to detect plasma protein leak (Brydon *et al* 1993) and bleeding into the gut (Brydon *et al* 1992), and for studies of mucosal cytokines (Ferguson *et al* 1994a), with minimal degradation of cells and molecules of interest.

The results presented in chapter XII show that peroral whole gut lavage can also be used to investigate inflammatory cells within the intestinal lumen. Studies of cells and their products can be combined with biochemical and immunological assays on WGLF, to provide detailed analysis and characterisation of immunological and inflammatory events in the gut. The capacity to investigate the relatively inaccessible small bowel is clearly advantageous, and, for research in IBD, measurement of IgG concentration in WGLF provides a simple and objective measure of disease activity. A heterogeneous group of IBD patients can thus be

subdivided and stratified into subsets with comparable disease activity, to allow investigation of other factors such as corticosteroid or diet treatments, or disease distribution (Ghosh *et al* 1994a). By applying WGLF analysis in other forms of gastrointestinal disease, it is possible to determine whether a particular immunological phenomenon is truly a feature of UC or CD, or is simply a non-specific expression of tissue injury in the gut.

Since it is already known that smears from centrifuged WGLF provide specimens for cytological diagnosis of colonic carcinoma (Rosman *et al* 1994), and since neutrophils can be found in faeces of IBD patients (Saverymuttu 1983b,c) it is not surprising that inflammatory cells have been detected in WGLF samples. With the method used, gut luminal cells can be counted as well as characterised cytologically, and the results show high counts of luminal cells, mainly neutrophils, in most patients with active IBD. However, it appears that this luminal neutrophilia is not a feature of small bowel CD. Only one of our patients with ileal CD had a high WGLF cell count, and there were two associated, complicating factors, NSAID intake and an abscess communicating with the gut.

Only one study has reported that faecal excretion of radio-labelled leukocytes is much higher in colonic than in small bowel CD (Saverymuttu *et al* 1986b); in other reports, regional data are not presented (Saverymuttu *et al* 1986a, Saverymuttu *et al* 1983a), or there is no observed effect of regional distribution (Teahon *et al* 1991). Most of these reports concern small numbers of patients, as is the case with the present study, based on WGLF cytology.

Eosinophils, though detected less frequently in WGLF than neutrophils, were observed in WGLF from 6 of 15 patients with active IBD and from one patient with carcinoma complicating UC. In all of these patients, there were also many neutrophils in the WGLF specimens. Preliminary work on assay of a biochemical marker for eosinophils, eosinophil cationic protein, supports this finding (Croft *et al* unpublished data).

Further studies with WGLF may help to elucidate the stimuli for inflammatory cell migration into the gut, and local activation. Various substances may act as chemoattractants, including IL-8, bacterial peptides such as FMLP, leukotriene-B4, complement factors such as C5a, and platelet-activating factor (PAF) (Ghosh *et al* 1994a). In chapter XIV, I have investigated the role of IL-8.

16.2. Granulocyte elastase assay in whole gut lavage fluid

Cytology is time consuming and needs fresh specimens. Hence it is difficult to obtain data on large numbers of patients during the course of routine clinical investigations. Following on from the cytology of WGLF, I have evaluated a biochemical assay, specific for the neutrophil granule protein GE. Enzyme-linked immunosorbent assays for GE measure the enzyme as a complex with α-1-proteinase inhibitor (α-1-antitrypsin)(Neumann *et al* 1984). The concentration of α-1-proteinase-inhibitor in WGLF is normally very low and is affected by disease(Brydon *et al* 1993), and so an ELISA for GE was unsuitable for the assay. A different technique, independent of the presence or concentration of α-1-proteinase-inhibitor, was therefore used. GE was released from the cells by sonication, then assayed using a chromogenic substrate (L-pyroglutamyl-L-prolyl-L-valine-p-nitroanilide) specific for GE, and a colorimetric method. Free GE in the fluid can also be measured, in pre-filtered specimens, thus providing further information on cell activation.

The present study shows that WGLF GE concentration significantly correlates with cell count, confirming that GE is an excellent surrogate marker for luminal neutrophilia. Results of GE assays in this large series confirm and extend our cytological findings, that most, but not all, patients with luminal neutrophilia have IBD. When stratified separately by disease distribution (based on X-ray and endoscopic findings) and by disease activity (based on concentration of IgG in WGLF), it emerges that luminal neutrophilia is a feature of active UC and active colonic CD but is not normally present in patients with active CD of the small bowel. This confirms the results of cytology.

There is an alternative although unlikely explanation of the regional differences in WGLF GE concentration in active disease, namely that there is a difference in the relationship between WGLF IgG concentration and disease

activity (as measured, say, by CDAI) if small bowel and colonic disease are considered separately. Re-examination of the raw data from my predecessors's earlier report does not support this (Choudari *et al* 1993).

Neutrophils and GE are also present in the WGLF from patients with diseases other than IBD, particularly in those whose clinical background was compatible with neutrophil migration through the gut wall. Acute radiation proctocolitis and diverticulitis are conditions obviously associated with pus present on the mucosa. A pericolic abscess communicating with the lumen may discharge pus, which is the likely explanation in our patient with carcinoma of the colon with elevated GE. Non-steroidal anti-inflammatory drug (NSAID) use has been shown to be associated with isotope-labelled leukocyte excretion into the lumen, probably from small intestine (Bjarnason *et al* 1987); two of our patients with markedly raised WGLF GE but normal IgG concentrations, were taking NSAIDs. The gut bacterial flora may have a role in NSAID enteropathy, as there is evidence that the neutrophil chemoattractant may be a metronidazole-sensitive micro-organism (Bjarnason *et al* 1992). However, metronidazole may affect neutrophil chemotaxis by inhibiting endothelial cell adhesion as shown in rat models, independent of its antimicrobial action (Arndt *et al* 1994).

The patient with active colonic CD who had undetectable GE in WGLF deserves further comment. This 25-year-old British man had developed diarrhoea while working in India, and had received prolonged courses of tetracycline to treat what was presumed to be tropical sprue before the diagnosis of CD was made. Another patient with active perianal CD and undetectable GE had received prolonged courses of metronidazole. Effects of antibiotic treatment on luminal neutrophils and luminal chemo-attractants will be a fruitful area for further research.

The relationship between disease activity (IgG in WGLF or the physician's global score) and WGLF GE was not so clear-cut in UC patients. All those with active UC and undetectable GE were receiving systemic or local steroids, which presumably modified their inflammatory immune response, though no significant effect of steroid therapy could be detected when analysing the whole group of IBD patients. Serial studies with whole gut lavage to assess the response to treatment (which are in progress) may clarify the effect of steroid treatment better than these cross-sectional studies and, indeed, preliminary unpublished data for CD show that WGLF IgG and GE do not diminish at comparable rates when patients are treated

with systemic steroids. In the present series, six of the 15 patients with inactive UC had detectable GE in WGLF, three of whom were on systemic steroids. This reinforces the difference between clinical definition of activity and the objective measure of inflammation provided by a marker of neutrophils. Measurement of specific parameters of mucosal inflammation may allow much better definition of full remission.

There were a few IBD patients in whom results of total and free GE assays showed the presence of both cell-bound and cell-free GE in WGLF, indicating neutrophil degranulation; other IBD patients had cell-bound (or, strictly speaking, particulate) GE only. Total GE levels were significantly higher in the former group, but disease activity, as measured by WGLF IgG concentration, was similar in those with and without free GE. I had expected that free GE would not be present in steroid-treated patients, because of the stabilising effect of these drugs on cell membranes, but in fact the opposite was found. These data can be explained within the framework which is being promoted by our institution (Ferguson *et al* 1994a), that IBD "illness" is due to a combination of inflammatory, destructive, infectious and other factors. Thus although a group of steroid-treated patients have a comparable level of disease activity to a group of non-steroid-treated patients (by CDAI, global assessment or WGLF IgG concentration), there may remain striking differences in overall illness severity, as reflected in our patients by luminal neutrophilia.

The effect of corticosteroids on luminal migration of neutrophils has not been well investigated in longitudinal studies and hence it is not known whether steroids may actually promote neutrophil migration into the lumen when the disease is still active. As emphasised above, it is probable that WGLF IgG and GE are in fact dissecting different facets of inflammation and giving different information about the inflammatory state of the mucosa.

16.3. Investigation of proinflammatory cytokines in WGLF

The role of cytokines in intestinal inflammation can be investigated by a variety of techniques. The cytokine protein may be directly detected by ELISA or its biological effects may be measured by bioassay. Bioassays are unsuitable for measurement of gut-derived cytokines and this has been shown by previous work in our laboratory - the bioactivity of these proteins is often lost in the gut lumen as a

result of enzymatic action. Gut tissue homogenates (Brynskov *et al* 1992a) or isolated mucosal mononuclear cells (Mahida *et al* 1989, Pullman *et al* 1992) in culture are often used to detect cytokine proteins by ELISA.. The cytokine message may be detected by *in-situ* hybridization (Cappello *et al* 1992) using nucleic acid probes. This is useful if the protein is quickly destroyed *in-vivo,* and this method is also widely used for cellular localisation of the production of these cytokines. Expression of the cytokine gene or tiny amounts of message may also be detected by the polymerase chain reaction (PCR) (Isaacs *et al* 1992, Izutani *et al* 1995) or reverse-transcriptase PCR (Eckmann *et al* 1993). Access to tissues is generally needed for all the above methods, thus limiting their application to when surgery or colonoscopy is performed. Investigation of the role of cytokines in small bowel disease is difficult unless the patient had a surgical resection. Data obtained from resection specimens are subject to a number of confounding variables such as the duration of inflammation, prior medical therapy, complications such as abscess formation and the surgical manipulation itself and hence must be interpreted with great caution. Though colonic disease is accessible to colonoscopy, this is an invasive procedure and not particularly suited for research use. In CD, patchiness of involvement leads to sampling bias and the bowel preparation technique may influence results. Even in colonic disease, serial studies to investigate changes resulting from therapy are virtually impossible., and establishing a baseline prior to modification by therapy may be difficult. A particular problem has been in obtaining 'real' healthy controls - for ethical reasons this is hardly ever possible.

Some of this drawbacks may be circumvented in clinical practice by the use of the WGLF analysis technique. Work done my predecessor (Mwantembe, Ph.D. thesis, 1992, University of Edinburgh) had shown that cytokines such as TNF-α or their receptors such as IL-2R could be detected in WGLF. In this thesis I have concentrated on IL-1β and IL-8, IL-6 and IFN-γ. Interest in IL-8 was a logical continuation of the previous work on neutrophil migration, as IL-8 is a potent, specific neutrophil chemoattractant (Baggiolini *et al* 1989) and is probably the first neutrophil chemoattractant to be produced by activated mononuclear cells as a result of immune activation (Harada *et al* 1994). IL-1β is one of the cytokines stimulating mononuclear cells to produce IL-8. Furthermore, as discussed in chapter II, IL-1β is an important cytokine involved in metabolic alterations in inflammation and attempts to measure IL-1β in serum in IBD by other workers have

been consistently disappointing (Satsangi *et al* 1987, Brynskov *et al* 1991). IL-6 may act as an important second messenger of IL-1 and TNF (Brynskov *et al* 1992b) and IFN-γ activates several important inflammatory cells.

Analysis of WGLF showed that the concentration of the proinflammatory cytokine IL-1β was significantly raised in active IBD. The role of IL-1β in intestinal inflammation and metabolic abnormalities has been reviewed in chapter II. An interesting observation was the detection of IL-1β in WGLF in almost half of the patients with inactive CD and in two-thirds of the patients with inactive UC. The median concentration in inactive IBD was significantly lower than in active IBD. This shows a dissociation between clinical remission in disease activity (which is indicated by a WGLF IgG concentration <10μg/mL) and immunological events in the gut mucosa. Previous studies using ^{111}In-labelled leukocytes had demonstrated significant leukocyte migration into the gut lumen in inactive CD (CDAI < 150) (Saverymuttu 1986b) and this was confirmed by analysis of GE in lavage fluid as described in chapter XIII. Detection of IL-1β was not specific to IBD as other inflammatory and neoplastic diseases of the gut such as radiation enterocolitis, infective colitis, ischaemic colitis, NSAID enteropathy and carcinoma of the colon also had detectable IL-1β. However, only 4 of these patients (with radiation colitis and carcinoma of the colon) had an IL-1β concentration greater than 100pg/mL, which was commonly found in active IBD. There was no difference in IL-1β between small bowel and colonic CD. Those on systemic steroids had a similar concentration to those not on steroids for a given disease activity.

IL-8 concentration in WGLF was significantly increased in active IBD but not in inactive IBD. Detectable IL-8 levels were present in only about half of the patients with active IBD. There was no difference in IL-8 levels between small bowel and colonic CD and those on systemic steroids had a similar concentration to those not on steroids for a given disease activity. Detectable IL-8 concentrations were present less frequently in the diseased controls; patients with radiation colitis, carcinoma of the colon and microscopic colitis had detectable WGLF IL-8 and these diseases were characterised by luminal neutrophilia.

The mechanism for neutrophil migration into the gut lumen in intestinal inflammation is likely to be multifactorial. IL-8 concentration in WGLF is likely to reflect luminal concentration, though it is obviously diluted by the lavage.The finding that increased amounts of IL-8 in the lumen were accompanied by

increased number of neutrophils as measured by GE concentration in WGLF in UC makes IL-8 a possible factor involved in luminal migration of neutrophils. This is in accordance with rectal and sigmoid perfusion studies which found that IL-8 concentration in the perfusate correlates with myeloperoxidase released from neutrophil granules in UC (Raab *et al* 1993). In contrast, in CD, there was no correlation between IL-8 concentration and GE concentration - this was true both for small bowel and colonic involvement. This suggests that the mechanism of neutrophil migration into the gut lumen in UC and CD might be different. In CD, luminal neutrophilia was mainly a feature of colonic CD but not small bowel CD. A luminal chemoattractant such as bacterial peptide with an aboral gradient might be involved in CD. Further support for this hypothesis comes from the finding that neutrophils were often absent from the gut lumen subsequent to antibiotic therapy as mentioned above. It is noteworthy that luminal neutrophilia in NSAID enteropathy was unaccompanied by any increase in IL-8 concentrations - as mentioned above, there is evidence that neutrophil migration in this condition is due to a metronidazole sensitive microbe (Bjarnason *et al* 1992). Teahon *et al* 1993 has provided evidence, based on radiolabelled red and white cell scintigraphy, that the mechanisms of neutrophil migration in CD and UC might be different - in CD, an intraluminal signal may be involved whereas in UC, the neutrophils are activated and degranulated within the mucosa. Furthermore, neutrophil migration in NSAID enteropathy may have a similar mechanism to CD. The findings presented in the previous chapters would provide further support for this hypothesis.

IL-1β is a potent inducer of IL-6 in both macrophages and T-cells (Bendtzen 1991b). Colonic mucosal IL-6 has been reported to be increased in IBD (Mitsuyama *et al* 1991). However, IL-6 was infrequently detected in WGLF. The short half-life of IL-6 (5 minutes) might be one possible explanation (Brynskov *et al* 1992b). IFN-γ is a lymphokine that acts as a potent activator of macrophages (Bendtzen 1991a). IFN-γ too was infrequently detected in WGLF. IFN-γ production in the inflamed mucosa has been variably reported by workers as normal (MacDonald *et al* 1990), decreased (Lieberman *et al* 1988) or increased (Fais *et al* 1991), and methodologic differences are likely to be responsible for the discrepancies.

One of my reasons to explore methods of measuring gut derived cytokines was to investigate their role in producing nutritional abnormalities, such as

osteopenia. The results presented in chapter XIV did not show any difference between CD and UC in WGLF IL-1β or IL-6 concentrations. It may be seen from table 14.11 that, even in untreated patients, the concentration of WGLF IL-1β was similar in CD and UC. This was true for the other cytokines measured, such as IL-6, IFN-γ and IL-8. Work with TNF-α is ongoing, and has not been presented in this thesis. Though certain pro-inflammatory macrophage derived cytokines have been measured in this thesis, biological activity of cytokines is complex. IL-1 activity, for example, is modulated by two soluble IL-1 receptors and blockade of receptors by the naturally occurring IL-1-receptor antagonist (Dinarello and Wolff, 1993). Unless the relevant cytokine antagonists and soluble receptors are measured, the interpretation of results obtained by assaying a particular cytokine is limited. Further work is needed in analysis of WGLF by assaying, for example, IL-1-receptor antagonist and studying the IL-1/IL-1-receptor antagonist ratio. The work in this thesis should be considered descriptive, till such analytical data is obtained.

16.4. Growth factor peptides

Cytokines and growth factors secreted by immunocytes in the inflamed intestine can profoundly affect the activation state of mesenchymal cells, thereby amplifying the inflammatory response and probably contributing to fibrosis (Sartor 1994). IL-1 stimulates the proliferation of intestinal smooth muscle cells and fibroblasts. IGF-1 and TGF-β stimulate proliferation of fibroblasts and induce collagen synthesis by fibroblasts and smooth muscle cells. WGLF IGF-1 was detectable in patients with CD, often associated with strictures, but was undetectable in UC except in an atypical patient with long-standing active disease on cyclosporin. IGF-1 was present in both active and inactive CD patients. Other patients with detectable IGF-1 in WGLF had conditions known to be associated with increased fibrogenesis, such as post-radiotherapy, ischaemic colitis, diverticulitis, microscopic colitis, pouchitis and carcinoma of the colon. TGF-β_1, on the other hand, was detectable in all WGLF samples and did not discriminate between CD and UC. TGF-β is a dominant endogenous factor in promoting reconstitution of the integrity of the epithelium (Montesano *et al* 1988), and the reduction seen in patients with previous or current radiotherapy may impair such healing.

16.5. Conclusions

In conclusion, WGLF analysis is a powerful technique to investigate immunoinflammatory events in the gut in large numbers of well-stratified heterogeneous patients. Neutrophil migration may be directly quantitated using a simple assay and chemoattractants involved in neutrophil migration may be investigated. Proinflammatory cytokines such as IL-1β which initiate a cascade of cellular activation may also be measured. It is clear that most of the abnormalities detected are not specific for IBD but may be found in other inflammatory conditions of the gut. However, this technique has permitted the reporting of previously unrecognized differences between small bowel and colonic CD disease and in the mechanism of neutrophil migration in UC and CD.

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EPILOGUE

My work described and discussed in this thesis not only confirms that Crohn's disease (CD) is associated with increased nutritional morbidity, but also presents evidence that direct effects of the disease may be important in causing specific nutritional disorders such as low bone mineral density. Body composition changes are in keeping with the hypothesis that specific metabolic derangement occur in CD. These findings support the concept of CD as a systemic disorder with a long premorbid period and distinguish it from ulcerative colitis (UC), a local mucosal disease. Furthermore, children and adolescents have been identified as a specially vulnerable group for osteopenia in addition to growth and developmental delay. Having completed the phase of work described and in parallel with writing up this thesis, I am now embarking on a programme of work to take further the issues and concepts I have developed.

(1) A prospective study to address the question of osteopenia in children and adolescents with IBD is in preparation. The severity and prevalence of this problem would be investigated in a large cohort. Patients with spine Z-score lower than -1.0 would be randomised to one of two intervention groups: a programme of physiotherapy Vs physiotherapy and vitamin D/calcium supplements.

(2) It has recently become possible to investigate bone metabolism by measuring reliable and sensitive markers of bone turnover. A collaboration has been set up with Dr Simon P Robins who heads the bone research group at the Rowett Research Institute, Aberdeen, to measure plasma and urinary markers of bone turnover in a new cohort of IBD patients at diagnosis. Preliminary results of this ongoing study are presented in chapter VI. The findings of this study may influence choice of potential therapeutic interventions.

(3) Though corticosteroid therapy is not the only factor causing osteopenia in CD, it is certainly contributory. In UC, it probably is the most important factor causing osteopenia. Locally active steroids with high first-pass metabolism, such as budesonide, have recently become available for treatment of IBD and a multicentre study is being launched to compare bone mineral loss after treatment with budesonide controlled-release capsules compared with conventional prednisolone therapy.

(4) Analysis of the laboratory studies done as a part of this thesis showed that whole gut lavage fluid (WGLF) analysis is a powerful tool to objectively measure immuno-inflammatory events in CD or UC. Neutrophil migration into the gut lumen was found to be a feature of colonic CD and UC but not of small bowel CD. IL-8 might be the relevant chemotactic factor for luminal migration of neutrophils in UC but, in CD, other chemoattractants with aboral gradient such as bacterial peptides are clearly worthy of further studies. There are various methods to study neutrophil chemotaxis *in vitro* (Zigmond *et al* 1986); the neutrophil polarisation assay has been developed by Professor Wilkinson's team in Glasgow as a useful, robust screening test for neutrophil activation and migration (Haston *et al* 1985). Studies have been set up in collaboration with Professor Wilkinson to assess the polarisation of neutrophils in the presence of WGLF from normals and IBD patients. This would aim to study the relative contribution of various chemoattractants including bacterial peptides in promoting neutrophil migration into the gut lumen.

(5) A further study is investigating the differences between small bowel and colonic CD for a given clinical disease activity using WGLF.

(6) Body composition analysis by a portable user-friendly machine was found to be a convenient and accurate method of measuring lean and fat compartments at the bedside. A cross-sectional study on a large cohort of IBD patients and healthy and diseased controls has recently been completed by a visiting dietitian which should provide further data. Preliminary analysis of this data clearly shows that underweight patients, who are mostly patients with CD, are heterogeneous regarding the proportion of lean and fat, unlike in normal weight patients where increasing body mass index is accompanied by an increase in percent fat. Undernutrition in CD is widely regarded as being multifactorial, but the contribution of the various factors, such as malabsorption, reduced intake, inflammation, surgery and medical therapy may now be analysed in terms of body composition changes. Precise therapeutic interventions for nutritional support will depend on understanding these complex pathogenetic issues.

It is hoped that this work and the methods developed will link with other developments by the members of the research group, including studies of cytokines, other inflammatory mediators and fibrogenesis.

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APPENDIX

Published papers

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GASTROENTEROLOGY 1994;107:1031–1039

Low Bone Mineral Density in Crohn's Disease, but Not in Ulcerative Colitis, at Diagnosis

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Background/Aims: The pathogenesis of low bone mineral density in patients with inflammatory bowel disease is unclear, and the relevance of secondary osteopenic influences is controversial. Our aim was to study bone mineral density in newly diagnosed patients. *Methods:* Bone mineral density and biochemical parameters of bone metabolism were measured in 15 patients with Crohn's disease and 15 patients with ulcerative colitis, all of whom were newly diagnosed. Lumbar and forearm bone mineral densities were measured by dual energy x-ray absorptiometry, and Z scores were obtained by comparison with age- and sex-matched normal values. Twenty-three patients had repeat measurements 1 year later, and 20 had received systemic steroids. *Results:* At diagnosis, the mean Z score for patients with Crohn's disease (spine, -1.06 ± 0.86; forearm, -1.04 ± 0.86) was significantly lower than that for patients with ulcerative colitis (spine, -0.03 ± 1.16; forearm, 0.11 ± 1.24). Inflammatory activity, disease localization, body mass index, smoking habits, sex, physical activity, or biochemical parameters did not account for this difference. Spine and forearm Z scores were significantly correlated. Mean Z scores after 1 year were not significantly different from initial Z scores. *Conclusions:* At diagnosis, low bone mineralization is a feature of Crohn's disease but not ulcerative colitis. Treatment with corticosteroids did not result in further bone loss in 1 year.

Low bone mineral content (BMC) has been convincingly shown in unselected groups of patients with inflammatory bowel disease (IBD),[1–4] but the pathogenesis of the osteopenia is incompletely understood. No correlation between bone loss and serum parameters of bone metabolism has been shown.[2] Although the deleterious effects of corticosteroids on trabecular bone mass are well documented,[5,6] longitudinal studies in patients with IBD have failed to show any significant correlation between the rate of bone loss and prednisolone therapy.[7] However, conflicting evidence from cross-sectional studies indicated that cumulative corticosteroid use may be an important[8,9] or even the sole cause[10] of osteopenia in patients with IBD. This is contradicted by other cross-sectional studies.[11]

Various factors, such as small intestinal resection,[12] ileal involvement,[9] vitamin D deficiency,[13] smoking,[8] body mass index,[7] and height,[14] have been correlated with osteopenia in IBD, reflecting the heterogeneous nature of the patient cohorts studied by various workers. We still do not know the extent to which bone mineral loss in IBD occurs as an integral manifestation of the disease as distinct from the secondary osteopenic influences of corticosteroid therapy, surgical intervention, inactivity, malabsorption of calcium and vitamin D, or the cachexia of inflammation.

There has been no comparative study on bone mineralization in patients with Crohn's disease (CD) and ulcerative colitis (UC). CD may be considered a systemic disease, whereas UC is more limited to colonic mucosal inflammation; CD also has important immunologic differences from UC.[15]

We have studied bone mineral density (BMD) in a series of newly diagnosed patients with CD and UC. Both spine and forearm BMDs were studied so that trabecular and cortical mineralization could be assessed because there are metabolic differences between these two types of bones.[16] Newly diagnosed patients were studied to avoid most of the secondary osteopenic influences mentioned previously. The measurement of BMD was repeated about 1 year after diagnosis to assess progressive bone loss and the effects of therapy.

Materials and Methods

Subjects

A total of 30 consecutive patients (17 male and 13 female) ranging in age from 14 to 83 years (median, 26 years) with newly diagnosed IBD were studied between March 1991 and August 1993. The diagnosis and disease extent in all patients were confirmed by histology, endoscopy, and radiology. In 15 patients (9 male and 6 female) ranging in age from 14 to 83 years (median, 24 years), CD was diagnosed. In

Abbreviations used in this paper: BMC, bone mineral content; BMD, bone mineral density; BMI, body mass index; DEXA, dual energy x-ray absorptiometry.

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Table 1. Characteristics of the Patients Recruited in the Study

	Male	Female
CD	9	6
Colonic	6	3
Ileal	1	2
Ileocolonic	1	1
Jejunal	1	0
UC	8	7
Pancolitis	4	3
Distal colitis	1	0
Proctitis	3	4

another 15 patients (8 male and 7 female) ranging in age from 14 to 63 years (median, 28 years), UC was diagnosed. The distribution of disease is given in Table 1.

None of the patients were pregnant, and none had known cholestasis, renal disease, hypogonadism, previous gastric surgery, or thyroid or parathyroid disorders.

The logistics of setting up BMD studies normally meant a delay of 2–3 weeks. For ethical reasons, we did not attempt to delay appropriate medical treatment during this interval. As a result, BMD measurements were made in 14 of the 30 patients shortly after the start of oral corticosteroids; in 13 patients, the duration ranged from 4 to 14 days and, in one case, 19 days.

Clinical Assessments

The patients were clinically assessed by one of the investigators (S.G.), and each patient completed a diary card for 1 week before BMDs for calculation of Crohn's Disease Activity Index (CDAI).[17] The hematocrit for CDAI was obtained from the full blood count. For patients with UC, both the CDAI and the Powell–Tuck index[18] were calculated. Smoking status, bone fracture history, and experience of contraceptive pill and hormone replacement therapy were recorded. The CDAI with or without the Powell–Tuck index were again calculated just before follow-up measurement of bone density. Height and weight were recorded, and the body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters).[2] The estimated duration of disease (in weeks) before diagnosis was also recorded. All patients younger than 20 years old had bone age assessed from a radiograph of the left wrist and hand (Greulich and Pyle).

Assessment of Physical Activity

The level of physical activity at the time of diagnosis and the premorbid level of physical activity when the patient was last free from IBD manifestations were recorded on a 1–5 scale as follows: (1) predominantly homebound, (2) walks once a week for shopping, (3) average activity (no formal exercise or hard work), (4) active (gymnasium, games, formal exercise at least once per week), and (5) regular athletic activities. The level of physical activity at the time of follow-up measurement of bone density was also recorded.

The scale of physical activity was validated against the Baecke habitual physical activity questionnaire, which is a reliable self-administered measure of physical activity.[19] Seven study patients, 10 patients with IBD not in the study, and 10 healthy volunteers completed the Baecke habitual physical activity questionnaire and were also graded on the scale given above by one of the investigators (S.G.). The age range of the participants was 18–57 years (16 men and 11 women). The Baecke questionnaire scores three dimensions of physical activity: work, sport, and leisure time. Grades 1 and 2, grade 3, and grades 4 and 5 significantly differed from each other in the mean scores of all of the three dimensions. Grade 1 significantly differed from grade 2 only in work score, and grade 4 differed from grade 5 only in sport score.

Bone Density Measurements

We used a Hologic QDR-1000/W (Hologic Inc., Waltham, MA) scanner to obtain dual energy x-ray absorptiometry (DEXA) measurements of lumbar L1–L4 vertebrae[20] and the right forearm (both radius and ulna). The Hologic forearm application protocol defines a global region of interest encompassing three individual forearm sites: (1) the one-third distal region is defined as a region 20-mm wide centered at a distance equal to one third of the forearm length measured from the distal tip of the ulna and contains mostly cortical bone; (2) the ultradistal region is 15 mm in length positioned proximal to the end plate of the radius, excludes the end plate of the radius, and contains mostly trabecular bone; and (3) the mid-distal region is the region between the one third and ultradistal regions and contains both trabecular and cortical bones. At our center, dominant and nondominant forearms were found to be comparable. The BMC divided by the area of interest gave the BMD in grams per centimeter square. The average BMD values for L1–L4 and the three forearm sites were used for calculations. Normal values for BMD readings with the Hologic QDR-1000/W scanner were available as mean BMD (±SD). The normal values for L1–L4 were available from age 0–85 years for both males and females; for forearm measurements, normal values were available from age 20–85 years for both males and females. The normal values were provided by the Hologic reference database obtained from healthy white volunteers of either sex (spine, 605 female, and 294 male subjects; forearm, 366 female, and 70 male subjects). None were taking steroids, anticonvulsants, fluoride, diuretics, or estrogens. Subjects older than 60 years of age had a lateral spine radiograph, and evidence of vertebral fractures or spine BMD below 0.82 g/cm^2 were considered as exclusion criteria. Statistical thresholds were set, and individuals below the first and above the 99th percentiles were excluded. Because the Hologic reference database is based on a United States population, we recruited 100 healthy normal volunteers from the local Scottish population. BMD values obtained from locally recruited control subjects were not significantly different from the Hologic reference database; hence, this database was used for our control values.

For scan-rescan measurements at our center, the coefficient

of variation for the lumbar vertebrae is 0.8%, and that for the forearm is 0.5%.

Z scores were calculated for each patient using the formula

$$Z \text{ score} = \frac{\text{Measured BMD} - \text{Normal BMD (age- and sex-matched)}}{\text{SD of the Normal BMD}}$$

Z scores were available for all BMDs from L1–L4; Z scores for the forearm could only be calculated for patients 20 years of age or older. In one 28-year-old man with UC, forearm BMD was not available.

Bone density measurements were performed after diagnosis and at follow-up 1 year later.

Biochemical Measurements

These were performed at the first assessment after diagnosis before measurement of BMD. These included (1) plasma calcium and urinary calcium/creatinine ratio measured in a 24-hour sample of urine collected in a special container containing HCl, (2) plasma phosphate and urinary phosphate/creatinine ratio measured in a 24-hour sample of urine, (3) plasma albumin, (4) plasma alkaline phosphatase, (5) plasma 25-hydroxyvitamin D, (6) plasma immunoreactive parathyroid hormone, and (7) urinary hydroxyproline/creatinine ratio measured in a 24-hour sample of urine collected in a special container with toluene.

Blood samples were taken after an overnight fast to avoid any influence of meals on serum phosphate concentration. 25-OH-D levels were measured throughout the year (4 patients in winter, 8 in spring, 8 in summer, and 10 in autumn) and compared with seasonally adjusted reference values. Plasma total calcium, phosphate, alkaline phosphatase and urinary calcium, and phosphate creatinine concentrations were measured by standard routine methods. Plasma immunoreactive parathyroid hormone was measured by immunoradiometric assay[21] (Nichols Allegro immunoradiometric assay; Nichols Institute Diagnostics, Geneva, Switzerland). Plasma 25-OH-D (D_2 + D_3) was measured by a competitive binding assay.[22] Urinary hydroxyproline was measured by colorimetric method.[23]

Statistical Analysis

Comparison between CD and UC was made using the unpaired *t* test. Comparison of initial measurements with follow-up measurements was made by the paired *t* test. Correlation coefficients were calculated with the Pearson's correlation test.

Ethical Considerations

The study was approved by the Medicine Subcommittee of the Lothian Area Ethics of Research Committee. Each patient gave informed verbal consent.

Results

The age and sex distribution, duration of symptoms before diagnosis, physical activity grade, and duration of steroid use before diagnosis for CD and UC are summarized in Table 2. The mean spine Z score was −0.56 (SD, 1.07) for 17 male patients and −0.48 for 13 female patients (SD, 1.25; P = NS). The mean of the 12 forearm Z scores available from male patients was not different from the mean of 9 forearm Z scores available from female patients (−0.41 ± 1.49 vs. −0.66 ± 0.99; P = NS).

Table 2. Clinical Features of CD and UC

Features	CD	UC
Age (*yr*)	24 (14–83)	28 (14–63)
Sex (M/F)	9/6	8/7
Estimated duration of disease before diagnosis (*wk*)	18.6 (18.6)	12.3 (15.5)
Duration of steroid use before BMD measurement (*wk*)	1.2 (1.1)	0.5 (0.8)
Grade of physical activity		
Premorbid	3.7 (0.6)	3.9 (0.3)
At diagnosis	2.8 (0.6)	3.1 (0.9)
BMI	21.38 (3.17)	22.36 (4.33)
Active disease		
CDAI > 150 or Powell-Tuck index > 4	11	10

NOTE. All values are expressed as mean (SD) except age, which is given as median (range).

The mean BMIs in patients with CD and UC are also given in Table 2. The spine and forearm Z scores obtained from BMC per unit area expressed as SD from the age- and sex-matched means were not significantly correlated with BMI (r = 0.20 and 0.11, respectively). However, spine and forearm total BMC significantly correlated with BMI (r = 0.76 and 0.75; $P < 0.05$), which implied that increasing BMI was associated with larger bones and, hence, a larger area of interest.

Four patients (2 girls with UC and 2 boys with CD) were younger than 16 years old. The patients with UC were on the 90th and > 97th percentile in height. The patients with CD were on the 90th and 3rd percentile in height. The 14-year-old boy with CD with height on the 3rd percentile had a bone age of 12. The bone age of the remaining three adolescents was appropriate for age. Four other patients (3 with UC and 1 with CD) were 18–19 years old, and none of them were short-statured (2 men with UC, 182 and 178 cm; 1 woman with UC, 157 cm; 1 woman with CD, 155 cm); their bone ages were that of mature adults.

The mean height of 7 men 20 years of age and older with CD (173 cm [SD, 4 cm]) was not different from the mean height of 6 men 20 years of age and older with UC (173 cm [SD, 5 cm]). The mean height of 5 women 20 years of age and older with CD (158 cm [SD, 6 cm])

was not significantly different from the mean height of 4 women 20 years of age and older with UC (166 cm, [SD, 4 cm]).

The mean CDAI in patients with CD was 196 (SD, 70). CDAI was calculated for patients with UC, and this was not different from that for patients with CD (mean, 164; SD, 105; P = NS). Eleven of the 15 patients with CD had a CDAI > 150, indicating active disease. Ten of the 15 patients with UC had a Powell–Tuck index >4, indicating active disease (Table 2). There was no correlation between the CDAI and spine or forearm Z score ($r = -0.17$ and -0.20, respectively) in patients with CD. Likewise, there was no correlation between spine or forearm Z score and the Powell–Tuck index ($r = -0.26$ and -0.22, respectively) in patients with UC.

Nine patients with CD had colonic involvement only, and six patients with CD had small bowel involvement. The spine and forearm Z scores in these two groups of patients are shown in Table 3. The Z scores for the two CD groups with different anatomic regions involved did not differ significantly.

Fourteen of the 30 patients were smokers, and the remainder were nonsmokers or ex-smokers. The mean spine Z score was −0.26 (SD, 1.15) in the 14 smokers and −0.74 in the 16 nonsmokers and ex-smokers (SD, 1.11). The difference was not significant. The forearm Z scores were available for 10 smokers and 11 nonsmokers and ex-smokers; the mean Z scores were not different (−0.58 ± 0.65 vs. −0.47 ± 1.51; P = NS). Among patients with CD, 8 were smokers; their spine and forearm Z scores were not different from those of the nonsmokers.

Two patients with CD were taking hormone replacement therapy for more than 5 years; their spine Z scores were −2.9 and −1.0. In neither case was bone disease the reason for prescribing hormone replacement therapy. Secondary amenorrhea was present in 1 patient with CD (spine Z score, −1.5) and 1 patient with UC (spine Z score, −1.1). Three patients with CD were taking oral contraceptives (spine Z scores, −0.6, 0.6, and −1.2), and 4 patients with UC were taking oral contraceptives (spine Z scores, −0.4, −0.5, 1.3, and −1.2).

Table 4 shows the biochemical parameters in CD and UC. None of the parameters were significantly different in the two groups of patients. Plasma Ca^{2+} levels were low in 2 patients with severe UC, both of whom had low plasma albumin levels; corrected Ca^{2+} levels were normal. Although urinary hydroxyproline excretions were above the upper limit of normal (168 μmol · 24 h^{-1} per square cm^{-1} of body surface) in 8 of the 30 patients, urinary hydroxyproline/creatinine ratios were within normal limits in all patients.

Table 3. Z Scores in CD According to Region of Involvement

	Colonic (Mean [SD])	Small bowel (Mean [SD])
Spine Z score	−1.09 (1.12) (n = 9)	−1.02 (0.41) (n = 6)
Forearm Z score	−0.86 (0.67) (n = 8)	−1.35 (1.17) (n = 4)

Table 4. Biochemical Markers of Calcium Homeostasis

Bone biochemistry (normal range)	CD (Mean [SD])	UC (Mean [SD])
Total calcium (*mmol/L*)* (2.25–2.50)	2.29 (0.04)	2.30 (0.04)
Urinary Ca/creatinine	0.26 (0.20)	0.29 (0.39)
Plasma PO_4 (*mmol/L*) (0.87–1.45)	1.11 (0.16)	1.22 (0.17)
Urinary PO_4/creatinine	0.50 (0.29)	0.80 (1.12)
Urinary OH-proline/creatinine	16.07 (4.46)	15.93 (3.88)
25-OH D (*nmol/L*)	36 (11)	41 (13)
Summer (15–100)	45 (12)	47 (14)
Winter (15–50)	28 (10)	37 (11)
Alkaline phosphatase (*U/L*)	137 (72)	99 (51)
Adult (30–140)		
Children (250–800)		
Immunoreactive parathyroid hormone (*ng/L*) (10–55)	39 (15)	41 (17)

*Total calcium corrected for albumin where appropriate.

Bone Density of Lumbar Spine

Figure 1 shows the lumbar BMD values related to age in patients with CD and UC separately for males and females. The mean spine BMD (0.88 ± 0.17) in patients with CD was significantly less than that in patients with UC (1.03 ± 0.14; $P < 0.02$). The rest of the comparisons between CD and UC are based on Z scores because these are indexed against age- and sex-matched controls.

Figure 2 shows the Z scores of lumbar spine for patients with CD and UC. The mean spine Z score for patients with CD was −1.06 (SD, 0.86), whereas the mean Z score of patients with UC was −0.03 (SD, 1.16). This difference is significant ($P < 0.02$). The mean Z score of the seven patients with proctitis was 0.16 (SD, 1.2), and this was not different from the mean Z score of the eight patients with more extensive UC (−0.2 ± 1.17; P = NS).

The 14-year-old boy with height on the 3rd percentile had his spine Z score recalculated using his bone age. This altered his spine Z score from −1.5 to −0.5 but did not alter the significance level of the difference between CD and UC ($P < 0.02$).

Bone Density of Forearm

Figure 3 shows the forearm BMDs for patients with CD and UC separately for males and females. Fore-

arm Z scores were available for 12 patients with CD and 9 patients with UC who were 20 years of age or older (Figure 2). The mean forearm Z score for patients with CD was −1.04 (SD, 0.86), whereas the mean forearm Z score for patients with UC was 0.11 (SD, 1.24). This difference is significant ($P < 0.05$).

The three different measurement sites of the forearm were separately analyzed as shown in Table 5. The Z scores for all three sites were significantly lower for patients with CD compared with patients with UC.

Relation Between Forearm and Spine Bone Density

Forearm Z scores were available for 21 of the 30 patients studied. The forearm Z scores of these patients were significantly correlated with spine Z scores ($r = 0.52$; $P = 0.02$) (Figure 4).

Follow-up

Follow-up data could be obtained in 11 of the 15 patients with CD. All of them had received systemic steroids. In addition, four patients had received rectal steroids. The mean cumulative corticosteroid dose between the initial and follow-up BMD measurement was 2.82 g (SD, 3.75 g). One patient was receiving azathio-

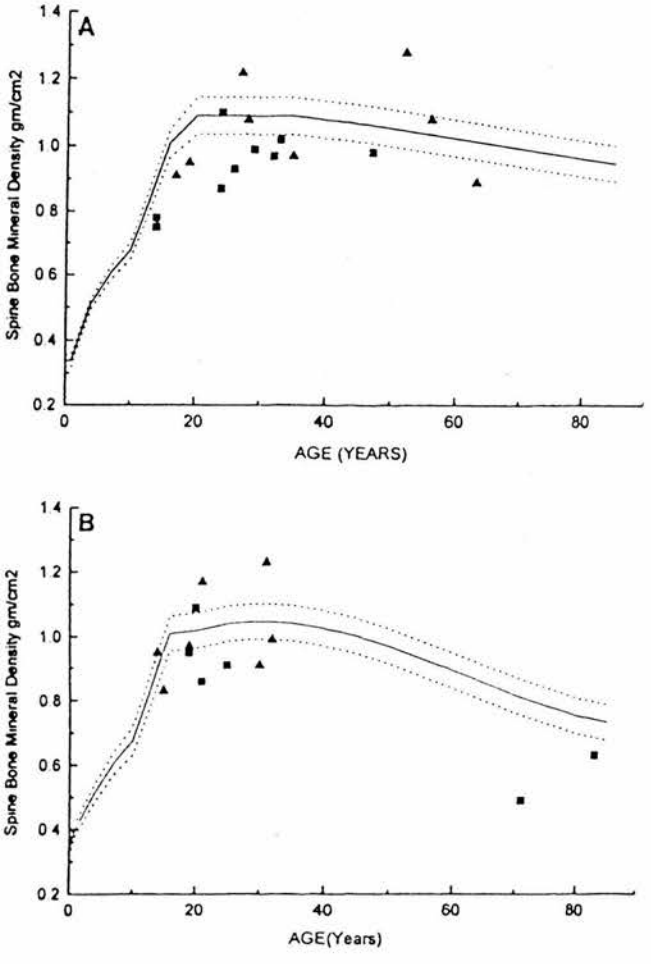

Figure 1. BMD of lumbar vertebrae L1–L4 in patients with IBD. (*A*) Male, (*B*) female. ■, CD; ▲, UC; ⋯⋯, mean ± 1 SD in normals.

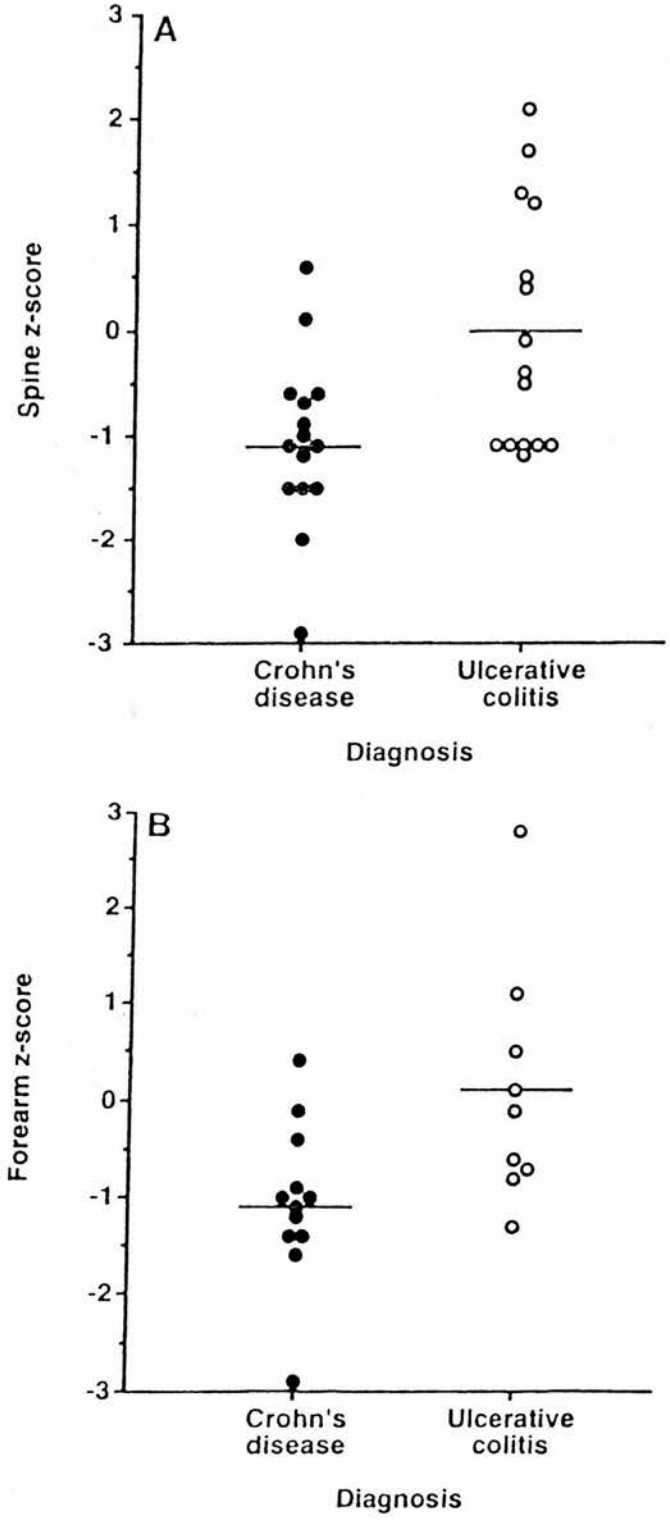

Figure 2. BMD Z scores in patients with CD and patients with UC at diagnosis. (*A*) Lumbar spine; (*B*) forearm. The *horizontal bars* represent mean values.

prine. Two patients had resectional surgery within this period (colectomy, 1; ileal resection, 1). The mean CDAI (86 ± 58) had decreased significantly ($P < 0.02$). Only one patient had a CDAI above 150 at the time of follow-up. The mean physical activity grade at follow-up was 3.1 (SD, 0.8), and this was not significantly different from that at diagnosis. The mean BMI at follow-up was 21.45 (SD, 3.85), and this too was not significantly different from that at diagnosis.

In these 11 patients with CD, a repeat measurement of bone density was performed after 1 year (Figure 5). The mean spine Z score after 1 year was −1.37 (SD, 0.82), and this was not significantly different from the mean spine Z score at diagnosis. Forearm Z scores were available in 8 of these patients, and the mean score was −1.17 (SD, 0.95); this too was not significantly different from initial measurements. In the single patient with CDAI >150, spine and forearm Z scores at follow-up were −1.6 and 0.4, respectively, compared with −1.5 and 0.4, respectively, at diagnosis.

Follow-up data were available for 12 of the 15 patients with UC. Nine of the 12 patients with UC had received systemic steroids and rectal steroids, whereas the remaining 3 patients had received rectal steroids only. The mean cumulative systemic corticosteroid use during this period was 2.25 g (SD, 2.80 g). The mean physical activity grade was 3.4 (SD, 0.7), and the mean BMI was 22.50 (SD, 4.33); none of these was significantly different from the relevant value at diagnosis. Two patients underwent panproctocolectomy with ileoanal pouch during this period. The mean Powell–Tuck index had decreased to 3.4 (SD, 2).

Twelve patients with UC underwent repeat bone density measurements. The mean spine Z score 1 year after the initial measurement was 0.04 (SD, 1.06), and the mean forearm Z score (9 patients) was 0.11 (SD, 1.07). None of these were significantly different from previous measurements.

Both spine and forearm Z scores continued to be significantly lower in CD compared with UC ($P < 0.02$).

Table 5. Z Scores for the Three Forearm BMD Measurement Sites in CD and UC

Diagnosis	One-third distal (Mean [SD])	Middistal (Mean [SD])	Ultradistal (Mean [SD])
CD	−1.67[a] (0.77)	−1.43[b] (0.82)	−0.60[a] (0.98)
UC	−0.70 (1.00)	−0.32 (1.19)	0.87 (1.53)

[a] $P < 0.02$; [b] $P < 0.01$.

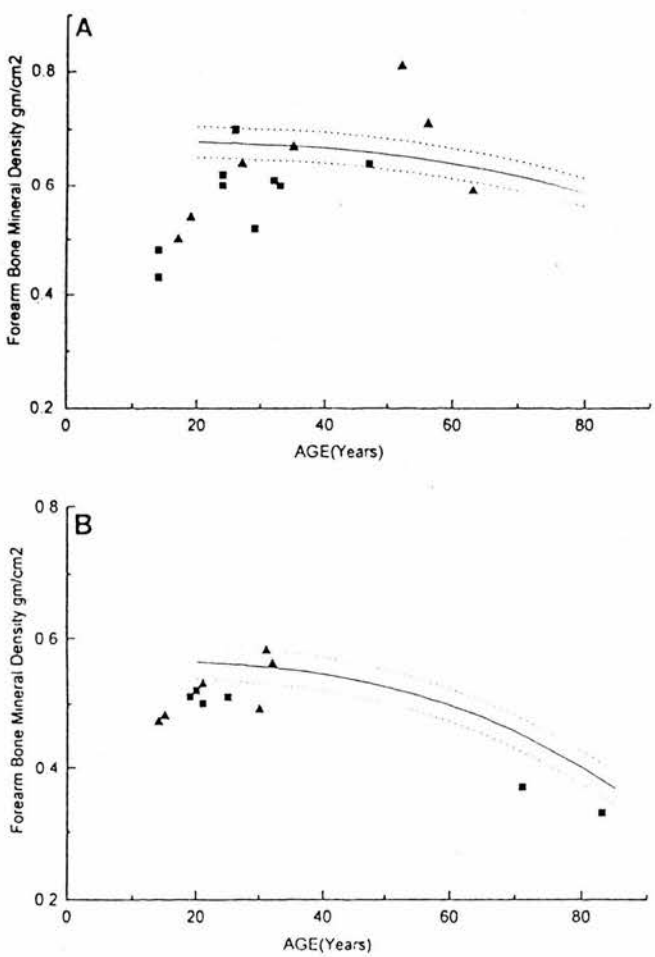

Figure 3. BMD of forearm in patients with IBD. (*A*) Male, (*B*) female. ■, CD; ▲, UC; ⁝⁝⁝, mean ± 1 SD in normals.

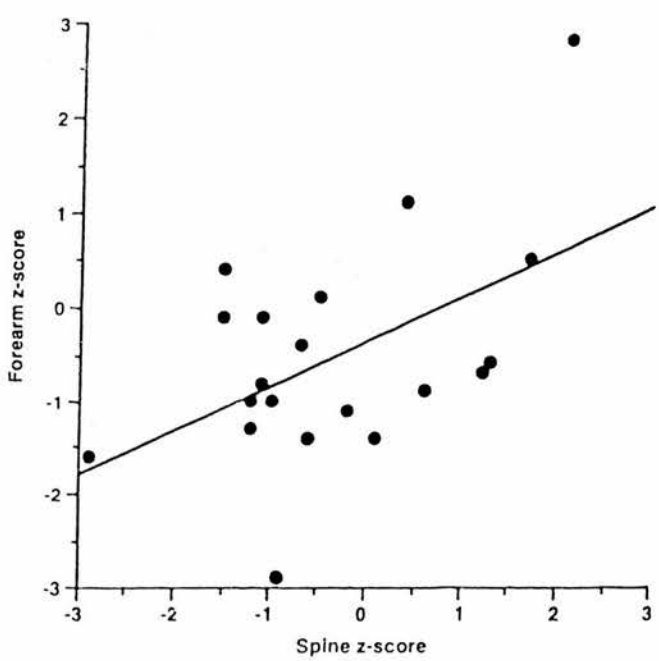

Figure 4. Correlation between forearm and spine bone mineral density Z scores in the 30 patients with IBD. $r = 0.52$; $P = 0.02$.

Discussion

We have not only confirmed many other reports of low bone mineralization in IBD but have clearly shown that at the time of diagnosis, this is a feature of CD but not UC. The two groups of patients were well matched with regard to age, sex, and severity and estimated duration of disease before diagnosis. The lower BMD in patients with CD applies equally to trabecular bone, comprising 50% of the lumbar spine,[20] and to cortical bone found in the forearm. This is not correlated with the CDAI or the BMI. Although physical activity was reduced at the time of diagnosis compared with usual activity levels, the grades of activity were similar for patients with UC and CD. Malabsorption is unlikely to be a major factor in the etiology of bone loss because patients with colonic disease had bone densities similar to those with small bowel involvement. This is further supported by the fact that bone metabolic parameters were normal and there were no differences between patient groups. However, calcium or vitamin D deficiency may exist in spite of normal biochemistry, and bone histology would be needed to definitely exclude this possibility. In view of the small numbers involved, the lack of difference in BMD Z scores between small bowel and colonic CD and between smokers and nonsmokers must be interpreted with caution because type 2 errors cannot be excluded. Further studies with a larger number of patients are needed before firm conclusions may be reached. Only 1 patient with UC was an ex-smoker. She had stopped smoking 3 years before presentation after smoking for 5 years. Her spine Z score was −0.5 and forearm Z score was 0.1. Excluding her from the nonsmoker group did not alter the statistical lack of significance between smokers and nonsmokers.

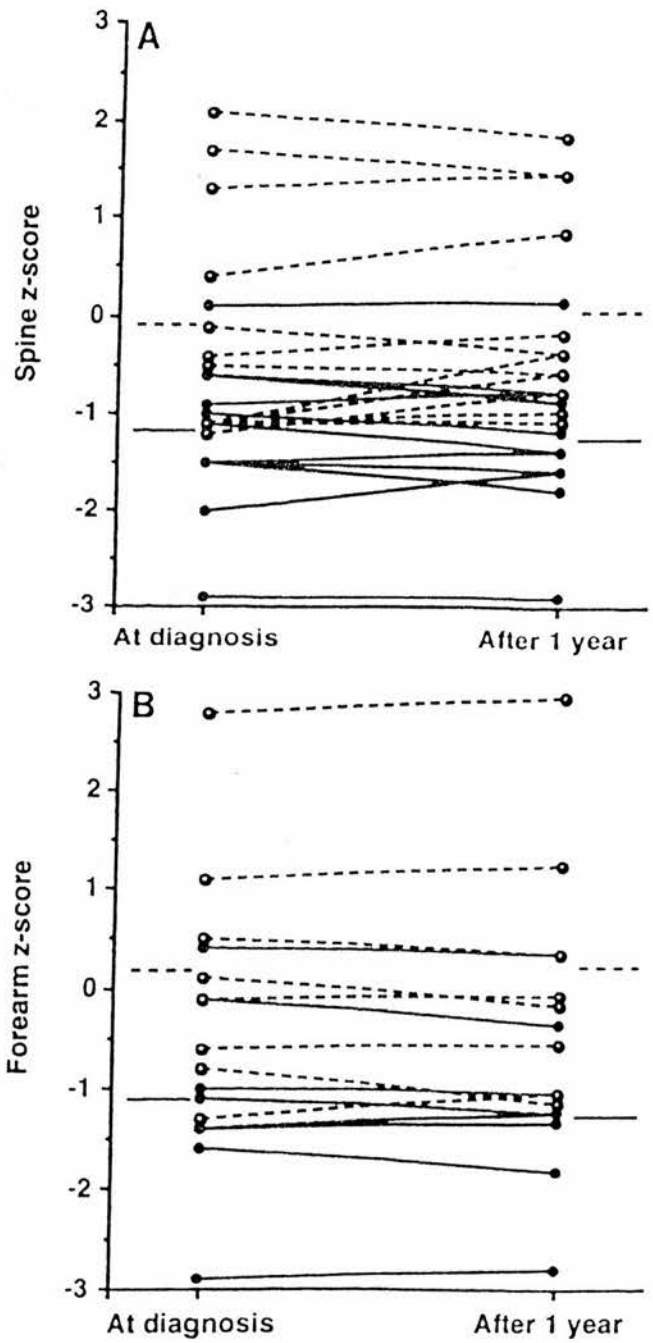

Figure 5. Comparison of BMD Z score values at diagnosis with Z score values at follow-up after 1 year. (*A*) Lumbar spine; (*B*) forearm. ——, CD; ---, UC. The *horizontal bars* represent mean values.

The results of this study underline the fact that CD and UC are different diseases. CD is a systemic disease with a long premorbid phase, whereas UC is a mucosal disease with an acute onset. We had previously shown in our study of Scottish children with IBD that 11 of 40 young children with CD were below the 3rd percentile for height, whereas none of the 14 children with UC was below the 3rd percentile for height.[21]

Maintenance of BMD should not be equated with the important issue of linear growth in growing children. The effect of IBD and its treatment on growth in children has been studied by various workers, including our group.[21] This study is predominantly on an adult population, and extrapolation to growing children should be performed with caution. Interpretation of BMD in adolescents needs additional considerations. Adolescents going through puberty at different ages may be an important confounding factor in interpreting serial BMD measurements, particularly if the patients are going through puberty at different ages from the control adolescents. In this study, however, only four patients (two with CD and two with UC) were going through puberty, and only one patient with CD had delayed puberty. All of the remaining patients were sexually mature adults. The patient with CD and delayed puberty was a 14-year-old boy with a spine Z score of −1.5 at diagnosis and

−1.8 after 1 year. Delay in growth may be a confounding variable if comparison is made with adolescents with normal growth. In this study, only the 14-year-old boy referred to above had a bone age less than chronological age with height on the 3rd percentile; recalculation of his Z score using bone age did not alter the significance level of the difference between CD and UC in spine Z scores. Because forearm Z scores were only available in adults 20 years of age or older, these results do not suffer from the confounding influence of growth and sexual development.

Despite the fact that 20 of 23 patients received systemic corticosteroid therapy, there was no evidence at 1-year follow-up of any further bone loss either in CD or UC. As a result of treatment, only two patients overall (one with CD and one with UC) had continuing active disease after 1 year; other series who report continuing loss of bone during follow-up have not been so successful in treating their patients.

Several studies have confirmed that osteopenia in patients with IBD is unassociated with calcium homeostasis defects.[1,2,7,10,11] Two recent studies reported as abstracts have recorded greater prevalence of osteopenia in CD compared with UC.[9,10] However, the two studies identify ileal involvement and greater steroid use, respectively, as the factors responsible for the difference from UC. None of these factors appeared to be relevant in our present study.

We failed to confirm the rapid bone loss reported previously[7] in some patients with IBD. In our patients, therapy generally results in control of activity of the disease with no further bone loss; indeed, a slight increase in Z scores occurred in some patients. Studies that have randomly recruited patients from the follow-up outpatient clinic for bone density studies are likely to be biased towards patients with complicated long-standing disease because they are likely to attend more frequently. For example, we have found low BMD in six teenagers with longstanding CD (spine Z scores ranging from −2.8 to −5.2). Two of these teenagers have sustained vertebral collapse. We reiterate that our follow-up period is only 1 year, and our conclusions should not be extrapolated to patients receiving more prolonged steroid therapy.

The mechanism of osteopenia in CD is unclear. The possibility that there is a primary disorder of osteogenesis so that bone formation is less than normal cannot be excluded. Abnormality of the mononuclear phagocytic system resulting in excessive resorption by osteoclasts or mediators from the inflamed gut triggering osteoclast activity are other possibilities that need investigation. Bone resorption is mediated by the unique multinucleated bone cell, the osteoclast, the formation and activity of which is regulated by a family of cytokines.[25] The cytokines that regulate osteoclast function include interleukin 1, tumor necrosis factor, transforming growth factor α, interferon gamma, interleukin 1 receptor antagonist, and interleukin 4.[26] Various cytokine abnormalities have been described in CD,[27,28] and differences from UC have been highlighted. Further studies are needed to elucidate the interaction of osteoclasts and cytokines in CD.

Our findings may have important clinical implications. It is likely that control of disease activity with appropriate therapy may actually improve bone mineralization, which may counterbalance the known osteopenic effects of steroid therapy. Effects of other interventions, such as regular exercise, calcium and vitamin D supplementation to offset gut losses, and hormone replacement therapy in postmenopausal women, need to be examined. Although our small numbers make it impossible to analyze the effects of secondary amenorrhea and hormone replacement therapy, our results show that it is not a confounding factor in explaining the difference between CD and UC. A recent study has reported prevention of bone loss in postmenopausal women by hormone replacement therapy.[29] To assess results of interventions, it is important to identify cases with intrinsic low bone mineralization and those at risk of accelerated bone loss.

We have found that BMD of lumbar vertebrae and forearm is reduced at diagnosis in patients with CD compared with UC. This is unrelated to abnormalities of calcium homeostasis, BMI, smoking, or prednisolone use. Treatment with corticosteroids does not necessarily result in further bone mineral loss.

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Prognosis in Nonalcoholic Steatohepatitis

Dear Sir:

We read with great interest the recent report on nonalcoholic steatohepatitis (NASH).[1] In their report, Bacon et al. describe the diversity of causes and outcomes of NASH and point out important clinical and pathological characteristics of the disease. However, prognosis and life expectancy still remains uncertain. In a recent study, we defined prognosis and life expectancy in 620 patients with chronic liver disease. After a follow-up period of 15 years (mean, 6.5 years), life expectancy was retrospectively calculated (Kaplan–Meier survival curves) and compared with an age- and sex-matched normal population (log-rank tests). Ninety-five patients (15%) (mean age, 46.8 years; 78% men) were found to have fatty liver disease. Diagnosis is based on clinical and, in 89%, on histological criteria. Sixty-five patients (68%) had evidence of alcoholic steatohepatitis, and 30 patients (32%) fulfilled the clinicopathologic criteria of NASH.[2,3] Five-year survival probability of patients with alcoholic steatohepatitis was 38%, and 10-year survival probability was 15%. In comparison, 5-year survival probability in NASH was 67% and 10-year survival probability was 59% ($P = 0.0001$). Patients with NASH had a lower life expectancy than the age- and sex-matched normal population; however, differences were not statistically significant.

Besides the divergent pathogenesis and clinical presentation of alcoholic steatohepatitis and NASH, life expectancy also differs significantly. However, progression of NASH seems to be slow because life expectancy was not statistically different compared with that of the normal population. Our data are in accordance with those of Bacon et al. and support the view that NASH should no longer be considered a largely benign and nonprogressive liver disease.[1] NASH includes a notable variety of potential causes and does not only affect obese women with diabetes. Further investigations are needed to elucidate the cause of NASH in each patient to offer a specific therapy presumably improving prognosis of the disease.

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Reply. We thank Propst et al. for their comments, which are in agreement with the findings and conclusions of our recent report on NASH. As they have pointed out, a major deficiency in our knowledge of this disease is the rate and frequency of progression of the disease and the long-term prognosis and survival. It will be possible to attain answers to these questions with longitudinal evaluation of a carefully described series of patients with NASH. More elusive will be an understanding of the underlying pathophysiological mechanisms of this disease, which will be required to provide logical and hopefully useful therapy to retard disease progression.

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Determinants of Bone Density in Inflammatory Bowel Disease

Dear Sir:

Ghosh et al. have reported that patients with newly diagnosed, untreated Crohn's disease have diminished bone density, whereas patients with newly diagnosed, untreated ulcerative colitis have normal bone density. Furthermore, in their study, once disease is treated with corticosteroids and rendered inactive, bone density remains unchanged and does not decrease further. The authors therefore suggest that disease diagnosis alone is critical to determining bone density and use their data to refute the notion that corticosteroid use is a key factor in determining the bone density of these patients.[1]

The clinical characteristics of the small groups tested necessitate that their data and conclusions for newly diagnosed patients with inflammatory bowel disease be corroborated. Twenty percent (3 of 15) of their patients with Crohn's disease were either prepubertal with a markedly abnormal bone age or postmenopausal. Only 40% (6 of 15) of patients with Crohn's disease had any evidence of small bowel disease. Table 3 of the report shows the differences between bone density in the colonic disease group compared with the small bowel disease group. Although the colonic group forearm bone density ($Z = -0.86$) is reported as not statistically different from the small bowel group forearm bone density ($Z = -1.35$), the apparent differences raise the possibility that a larger study might provide alternate results. I wonder how close this comparison came to the "magic" P value of <0.05? Nearly half (7 of 15) of the patients in the ulcerative colitis group only had ulcerative proctitis, potentially not providing as much of a systemically ill group of newly diagnosed patients with ulcerative colitis.

The main conclusion of this study was in contradistinction to the main conclusion of a study of bone density in patients with inflammatory bowel disease conducted by our group.[2] We found that the main predictor of decreased bone density was whether or not patients were using corticosteroids and was independent of disease diagnosis. With closer scrutiny, our two disparate conclusions may not be mutually exclusive. Ghosh et al. studied a group of newly diagnosed patients. Our study evaluated patients with a mean disease duration of Crohn's disease of more than 15 years and of ulcerative colitis of almost 9 years. Therefore, if both investigative groups are correct, it is possible that, at initial diagnosis, patients with Crohn's disease may have lower bone density than patients with ulcerative colitis; however, over time and with considerable corticosteorid use, the diagnosis no longer becomes a critical determinant and the daunted effects of corticosteroids on bone become paramount.

It is noteworthy that the Z score for spinal bone density of Ghosh et al. of patients with Crohn's disease was -1.06, whereas after several years of disease, the Z score for spinal bone density of our patients with Crohn's disease was a near identical -1.01 (using the same DEXA scanner; Hologic Inc, Waltham, MA). The group of patients with Crohn's disease of Ghosh et al. had a mean age of 24 years, including 2 postmenopausal women, implying that, overall, this was a very young group of patients. The mean age of the patients with Crohn's disease in our group was 33 years, and postmenopausal

Table 1. Spine Bone Mineral Density (Z Scores) in Patients With Long-standing Inflammatory Bowel Disease

Diagnosis	Age (yr) (range)	Sex (M/F)	Mean disease duration (yr)	Spine bone mineral density (mean [/SD])[a]	Z score
Crohn's disease (n = 10)	33 (20–58)	5/5	7	−1.17	(1.63)
Ulcerative colitis (n = 10)	38 (25–61)	6/4	7	−1.06	(0.46)

NOTE. Patients had inflammatory bowel disease for more than 5 years and at least 6 months of systemic steroids.
[a]Crohn's disease vs. ulcerative colitis; P = NS.

women were excluded. It is possible that having a systemic disease will have more exaggerated effects on the bones of younger patients who may not have completely established their adult bone density.

If it is true that, at initial diagnosis, patients with Crohn's disease have significantly lower bone densities than patients with ulcerative colitis, this does not necessarily imply a cause and effect relationship between developing Crohn's disease, having elevated circulating cytokines that may accelerate bone resorption, and the lowering of bone density. In fact, more than 25% of the patients with Crohn's disease in the study by Ghosh et al. had inactive disease at the time of study enrollment. It is possible that having Crohn's disease leads to an increased incidence of lactose intolerance in some patients, a decreased intake of high calcium containing foods, and, ultimately, diminished bone density. This could be tested by determining the calcium intake of those who present with low bone density compared with those who have normal bone density. Alternatively, these patients may have simultaneous genetically directed defects in the maintenance of normal intestinal and normal bone homeostasis.

Our group's data refute the notion that disease diagnosis affects bone density. We compared the small groups of patients who did not use corticosteroids with Crohn's disease (n = 9) and with ulcerative colitis (n = 7) and found no difference in bone density. Nonetheless, each study has asked similar questions of different stages of disease, allowing for both conclusions to be true. More data are required to sort through these differences, and, more importantly, an effective therapeutic approach at improving bone density is required.

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Reply. We thank Dr. Bernstein for his comments. We fully agree that osteopenia is a common complication of inflammatory bowel disease, but we suggest that it is likely to be multifactorial. Corticosteroids, malabsorption, dietary factors, and surgery are all highly relevant, but the abnormal bone mineralization that we have found in patients with Crohn's disease at the time of diagnosis indicates a primary contribution by the disease itself. Dr. Bernstein's data are in complete agreement with those of other investigators and with the interim results of a study in progress, summarized in Table 1, that there is significant osteopenia in patients with both forms of inflammatory bowel disease of long duration. We had found no significant change in bone mineralization at 1 year after diagnosis and had emphasized in the discussion of our report that these short-term results should not be extrapolated to a longer duration of follow-up.

It is indeed interesting that the median Z score for spine bone mineral density of our patients with Crohn's disease at diagnosis is virtually identical with that for Dr. Bernstein's group of patients with long-standing disease (mean disease duration, 15 years). However, because we have not seen Dr. Bernstein's data in full, we cannot comment on variables such as cumulative steroid dose, disease activity, and anatomy. In our experience, very low Z scores (< -3.0), although infrequent, seem to be a feature of long-standing, complicated Crohn's disease, particularly in adolescents. Our failure to detect a significant difference in bone mineral density between patients with colonic and small bowel Crohn's disease might be due to type 2 error, as we mentioned in our discussion. It would hardly be surprising if malabsorption due to small bowel disease contributed to osteopenia; however, this could not explain the overall differences between Crohn's disease and ulcerative colitis.

The osteopenic properties of corticosteroids are fully established; indeed, the main reason for our prospective study design was to control for this confounding variable. Our results add further support to the concept of Crohn's disease as a systemic condition with a long premorbid phase, whereas ulcerative colitis is of acute onset and normally localized to the colonic mucosa.[1] The fact that, in 4 of our newly diagnosed patients, Crohn's disease was clinically inactive (Crohn's Disease Activity Index <150) reinforces the importance of recognizing that inflammatory disease activity is only one component of inflammatory bowel disease illness. This has been clearly shown by our direct clinical studies of gut inflammation using whole gut lavage fluid.[2,3]

In this part of the United Kingdom, the prevalence of lactase deficiency in adults is less than 5%.[4] In any event, lactose intolerance is no more common in patients with Crohn's disease than in the general population[5] unless there is diffuse involvement of the small bowel, which was present in only 1 of our 15 patients. Bone mineral density results in patients with extensive colitis were similar to those with only ulcerative proctitis.

We agree that more data are needed. This is particularly relevant to the study of important subgroups such as adolescents and postmenopausal women. Furthermore, well designed studies on the pathogenesis of osteopenia are urgently needed before therapeutic strategies may be formulated.

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Neurobiology of Sleep and Human Colonic Motor Patterns

Dear Sir:

The relationship between sleep patterns and human colonic motor patterns[1] is supported by the association of poor sleep quality with the severity of irritable bowel syndrome symptoms in women with high anxiety and moderate depression.[2] The neurobiology is suggested by reports linking panic, wakefulness, and a shared smooth muscle disorder between the bladder and the bowel in young women to dopamine lateralized to the right hemisphere in which the metabolic rate is higher in women. This hypothesis is supported by subclinical impairment of lung airways and an abnormal parahippocampal region and its afferent and efferent connections in panic disorder.[3–7] It is also supported by diurnal variation of serotonergic-mediated inhibition of dopamine[8] leading to frequent propagating contractions in the morning that are likely to promote early-morning defecation[1] by monoaminergic interaction in the central nervous system[9] and by diurnal symptoms that patients with irritable bowel syndrome believe may be sleep-related.[2]

The fact that delay-dependent speeding of reaction time, indicating motor readiness, is abolished by depletion of dopamine[3] prompts the evaluation of adaptation to centrally mediated stimuli such as stress[10] by monitoring speech hesitation and switching pauses. This method is supported by the construct validity of response latencies, easily obtainable through microcomputer testing of spontaneous speech in emotionally charged dialogues, reflecting properties of neuronal activity and firing.[3,11]

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Is Aberrant Abnormal?

Dear Sir:

Thanks to the pioneering technique of Bird,[1] foci of colorectal mucosa that are positively identified by methylene blue staining are popularly called aberrant crypts nowadays, and there is almost a race to use this as a surrogate for cancer, i.e., an intermediate marker in evaluating everything from carcinogenesis to cancer prevention. In this regard, the editorial by Augenlicht[2] in the December 1994 issue of GASTROENTEROLOGY is most illuminating because of the timely contribution it makes in tying the molecular mechanisms of carcinogenesis with the morphological changes in colorectal cancer.

Some of Augenlicht's statements need to be corrected. "ACF were first reported by Bird in azoxymethane-induced mouse . . . "; not so, I submit, humbly. The foci of abnormal and dysplastic crypts in rodents were independently observed by Pozharriski, Ward, and Deschner around the same time in mid-1970s but first reported by Deschner in 1974.[3] That these are putative preneoplastic lesions in the human colon was observed (called abnormal, another word for aberrant as per Webster's dictionary!) by Shamsuddin et al. in 1981,[4] a decade earlier than what Augenlicht believes to be the joint discovery by Roncucci and Pretlow.[2] That these abnormal (or aberrant!) crypts do express carcinoembryonic antigen and p21 and $p21^{ser}$ (*Ki-ras*) was also reported first from my laboratory 8 years ago.[5] Aside from straightening the record, it is hoped that this communication would save the modern day investigators some time and effort in similar rediscoveries should they care to perform a literature search.

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Reply. Dr. Shamsuddin is correct that he, Deschner, and others made important and early contributions to our understanding of the histological changes in the normal appearing flat mucosa that precede

Analysis of Disease Distribution, Activity and Complications in the Patient with Inflammatory Bowel Disease

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Management strategies in Crohn's disease and ulcerative colitis should be based on up-to-date information on disease distribution, extent, activity and complications. A system of structured analysis is suggested, with separate consideration of destructive ulceration, inflammatory activity and other factors. Direct investigation of gut immunity by using whole gut lavage fluid (WGLF) is a valuable new technique of clinical investigation in IBD and related disorders. Recent studies have shown that the concentrations of plasma-derived proteins in WGLF provide objective measures of disease activity; and that this activity is a separate phenomenon from destructive ulceration and fibrosis. Neutrophils in the lumen can be investigated by cytology, or by assay of neutrophil elastase in WGLF. Cytokines and other immunoregulatory mediators can also be detected. These new techniques can provide a description of intestinal immunity and inflammation, based on a non-invasive test of 2–4 h duration. Work in progress shows that patients who respond clinically to elemental diet treatment have unusually high concentrations of soluble IL2 receptor in WGLF; cytokine profiles may facilitate the selection of patients suitable for other new treatment modalities.

Key words: Crohn's disease; cytokines; gastrointestinal protein loss; gut neutrophils; intestinal immunology; intestinal inflammation; ulcerative colitis

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What do physicians, gastroenterologists and society want and need in relation to IBD management? Most contributions to this Symposium volume concern techniques for assessment of the patient during an acute episode of illness. However, in the context of the long-term care of patients with IBD, it is important to widen the frame of reference.

Although epidemiological studies are difficult, there is general agreement that the incidence of Crohn's disease (but probably not of ulcerative colitis) is steadily rising. Scottish Hospital In-Patient Statistics (SHIPS) are collected prospectively on all admissions to NHS hospitals in Scotland, and by using record linkage the incidence of a disease rather than merely episodes of illness can be studied. We are using this powerful database to monitor trends in the incidence of juvenile-onset IBD. We recently reported that the recorded incidence of Crohn's disease in Scottish children, based on hospital admissions data, rose from 6.6 per million children in 1968 to 22.9 per million in 1983 (1). This trend continues, as shown in Fig. 1, with an unexplained excess of males in the period 1984–86. The overall incidence in 1988 was 29 per million children.

As with other chronic, treatable but incurable diseases, IBD patients deserve continuity of medical care at a single centre where multidisciplinary expertise is available for both assessment and management. Unless a cure for IBD is discovered, by the early years of the next century all of our specialist GI units will be caring for hundreds of patients, such as the woman whose life history is summarized in Table I.

STRATEGY FOR ANALYSIS OF OVERALL DISABILITY IN THE IBD PATIENT

Although general guidelines for the management of IBD patients can be presented in textbooks and journals, it must

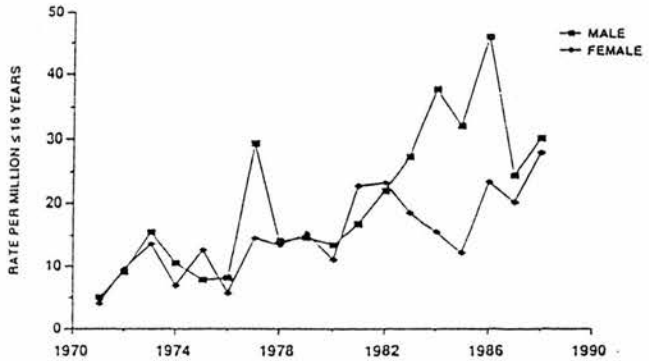

Fig. 1. Incidence of Crohn's disease in Scottish children; numbers of boys and girls having a first admission to an NHS hospital with a recorded diagnosis of Crohn's disease, per year.

Table I. Continuity of care during 50 years of Crohn's disease

Mrs F. born 1916
1934. vulval abscess
1937 diarrhoea? TB ileum: resection of ileum and ascending colon
1954 rectal stricture
1957 anterior resection: temporary colostomy, complications, total of four operations within a year: intermittent courses of corticosteroids in next 10 years.
1969 diarrhoea, recto-vaginal fistula, colectomy, ileostomy: thereafter, significant nutritional problems
1978 jaundice, cholecystectomy, stone in common bile duct
1984 sub-acute obstruction, division of adhesions
1987 died, bronchial carcinoma (lifelong smoker)

Treated at the Western General Hospital, Edinburgh for 50 years, by the following physicians and surgeons:

Mr Charles Illingworth (later Professor Sir Charles Illingworth)
Mr John Bruce (later Professor Sir John Bruce)
Dr (later Professor) Wilfrid Card
Dr Wilfrid Sircus
Mr (later Professor) Adam Smith
Mr Charles Falconer
Mr David Hamer-Hodges
Dr (later Professor) Anne Ferguson

be recognized that in individual cases management must be tailored to the patient's current anatomical, functional and disease activity status.

We recommend that an attempt be made to analyse, in a systematic way, the components of overall disability of patients with IBD, not only at presentation but during follow-up, and particularly when any change in therapy is being considered. A possible scheme is shown in Table II. This type of analysis can profoundly influence treatment plans. For example, it may be dangerous merely to increase the dose of corticosteroids if a patient's abdominal pain is, in fact, caused by local perforation and abscess.

In the last few years we have been developing a range of

Table II. A system for analysis of a patient with IBD

Extent and distribution of destructive chronic inflammation, i.e. macroscopically ulcerated, strictured or grossly inflamed segments.
Disease 'activity', i.e. inflammatory activity as assessed by fever, anorexia, tiredness, arthropathy, diarrhoea, pain.
Mechanical obstruction, which may be partly reversible if mucosal inflammation is a contributory factor.
Complications (e.g. abscess, fistulation between loops of gut to other organs such as the urinary tract or to the skin).
Nutritional status and related factors, e.g. anorexia, malabsorption, gut losses of blood and protein, and metabolic response to inflammation.
Iatrogenic factors both medical (e.g. steroid-induced diabetes) and surgical (e.g. bile salt and B12 malabsorption after resection of the terminal ileum).
Disease of other organs (related or unrelated to IBD) (e.g. duodenal ulcer, gallstones, ankylosing spondylitis).
Social and psychological factors affecting the patient and family.

Table III. Studies required for comprehensive investigation of the human GALT

1. Specific IgA antibodies.
2. Potentially immunopathogenic but also potentially useful IgM, IgG, IgE antibodies.
3. Antigen specific T cells (currently the only method available is based on intestinal antigen challenge, multiple biopsies and morphometric analysis of these biopsies).
4. T cell and macrophage activation by immunological marker studies or measurement of activation products.
5. Polymorphonuclear activation.
6. Immunoregulatory molecular signals.
7. Inflammatory cytokines.
8. Other molecular mediators of inflammation.

methods to allow clinical measurement of the various aspects of gut inflammation and immunity. These can now be used to assist the clinical analysis in IBD, and should be invaluable new tools for the objective evaluation of new forms of therapy.

INVESTIGATION OF THE HUMAN GUT IMMUNE SYSTEM

Ideally, in order to study the gut immune system in clinical situations, a range of components should be assessed (Table III). Animal and clinical studies show that, with very few exceptions, tests on components of the systemic immune system (blood antibodies and circulating cells and cytokines) are virtually useless as indices of mucosal immunity at gut level. Some general information on the function of the mucosa-associated lymphoid tissues can be obtained from studies of saliva or tears, but these materials cannot provide organ-specific information relevant to the gut.

There have been a number of studies on fluid aspirated from the proximal jejunum. If appropriately processed by addition of protease inhibitors, this is a valuable source of material. However, we now have good evidence that data based on analysis of faeces will be highly misleading, and despite the ease of specimen collection, such studies should be discouraged.

USE OF WHOLE GUT LAVAGE FLUID TO STUDY GUT LOSSES OF BLOOD AND PROTEIN, AND DISEASE ACTIVITY

The technique of whole gut lavage with non-absorbable polyethylene-glycol-based solution has been widely applied in clinical practice for cleansing of the bowel prior to barium enema, colonoscopy or colonic surgery. This is also a radical but excellent technique for the treatment of intractable constipation.

After gut cleansing is complete, the clear fluid passed per rectum, whole gut lavage fluid (WGLF) is essentially a whole gut perfusate. Our studies in adults and children have shown that this material can readily be used for biochemical and

immunochemical assays to assess intestinal immunity, inflammation and gut losses of protein and blood (2–10).

Measurement of GI blood loss

Occult GI bleeding can be measured by using a highly sensitive technique, HemoQuant, for assay of haemoglobin in WGLF (8). In patients with a normal GI tract, WGLF Hb concentration ranged from 0.5 to 5.1 μg/ml equating to an estimated daily occult blood loss of 0.1–1.2 ml. High values for WGLF Hb, indicative of chronic bleeding, were found not only in patients with colorectal cancer, but also in 7 of 16 patients with active inflammatory bowel disease (8).

GI protein loss—index of activity in IBD

Fluid obtained by whole gut lavage normally contains traces of IgG, albumin and α_1-antitrypsin. Normal values, based on results for 63 immunologically normal patients or volunteers are: IgG (by ELISA) <1–10 μg/ml; albumin (by immunoturbidimetry) <1–26 μg/ml; A1AT (by immunoturbidimetry) <1–19 μg/ml (7). In our initial technical appraisal of the technique we had found that higher concentrations of these proteins were found in WGLF from patients with IBD (2, 5). Our further experience has shown that assay of these proteins in WGLF cannot be used as a diagnostic test for IBD; normal results are obtained in some patients with unequivocal radiological or endoscopic abnormalities when disease is clinically inactive.

We carried out a prospective study of gut lavage in IBD patients (7) in whom disease activity was simultaneously assessed by using the Crohn's Disease Activity Index (CDAI) (11) or the Powell Tuck Index (PTI) (12). For IgG, concentrations in lavage fluid correlated closely with activity indices: in Crohn's disease, $r = 0.723$ ($p < 0.0001$), in ulcerative colitis, $r = 0.714$ ($p < 0.0001$). Results for lavage fluid albumin and α_1-antitrypsin concentrations were generally similar to those for IgG, but less sensitive in detecting active disease (Fig. 2).

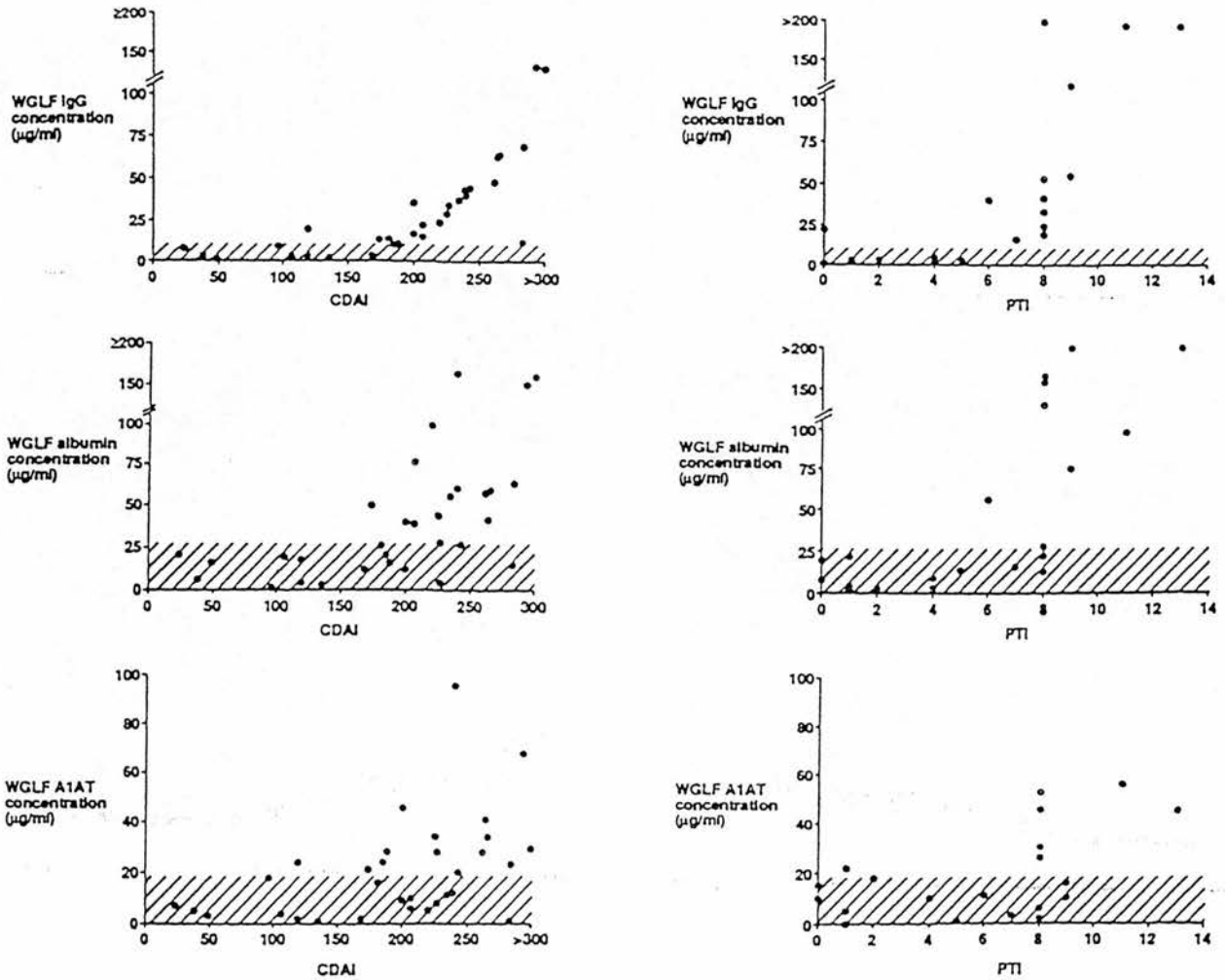

Fig. 2. Concentrations of IgG, albumin and alpha-1-antitrypsin in WGLF from patients with Crohn's disease and ulcerative colitis, plotted against CDAI and PTI, respectively.

Table IV. Concentrations of proteins in WGLF from patients with IBD: correlations with objective and subjective components of the CDAI and PTI (from further analysis of data in Fig. 2)

	Objective		Subjective	
	r	*p*	*r*	*p*
Crohn's disease				
WGLF IgG	0.452	0.003	0.623	<0.0001
WGLF albumin	0.462	0.002	0.524	<0.0001
WGLF A1AT	0.238	0.129	0.461	0.002
UC				
WGLF IgG	0.560	0.008	0.763	<0.0001
WGLF albumin	0.588	0.005	0.688	0.001
WGLF A1AT	0.429	0.052	0.545	0.011

Correlation between lavage protein concentrations and the objective and subjective components of activity indices

Surprisingly, for both CD and UC, concentrations of WGLF proteins correlated more closely with subjective parameters of the CDAI and PTI respectively (e.g. pain, malaise) than they did with objective parameters (Table IV).

Studies of luminal cells

Studies of radiolabelled cells have shown that there is increased faecal loss of leucocytes in active IBD. It is thus of interest to establish whether WGLF can be used in the investigation of gut leucocytes. By using Ficoll/Hypaque density gradients, we have found that cells can readily be separated from WGLF—these include squamous cells, enterocytes, neutrophil polymorphs, and also in some patients, eosinophils.

The enzyme neutrophil elastase is a specific marker for polymorphonuclear leukocytes. Table V shows results of assay for this marker carried out in WGLF from control subjects (in which only one of 32 specimens was above the level of detection, 0.03 per ml), together with non-IBD and IBD patients. The three non-IBD patients with high values all had abscesses communicating with the gut.

Table V. Concentration of neutrophil elastase in WGLF from a series of patients with GI disease, and controls

	Neutrophil elastase (units/ml)		
	<0.038	0.038–0.2	>0.2
Volunteers or trivial	31	1	—
Anaemia	33	1	2
Small bowel disease	23	—	—
Colonic disease	29	3	1
Miscellaneous	22	2	—
IBD: Crohn's active (IgG > 10 μg/ml)	12	—	16
Crohn's inactive	21	6	1
UC active (IgG > 10 μg/ml)	3	—	4
UC inactive	12	3	1

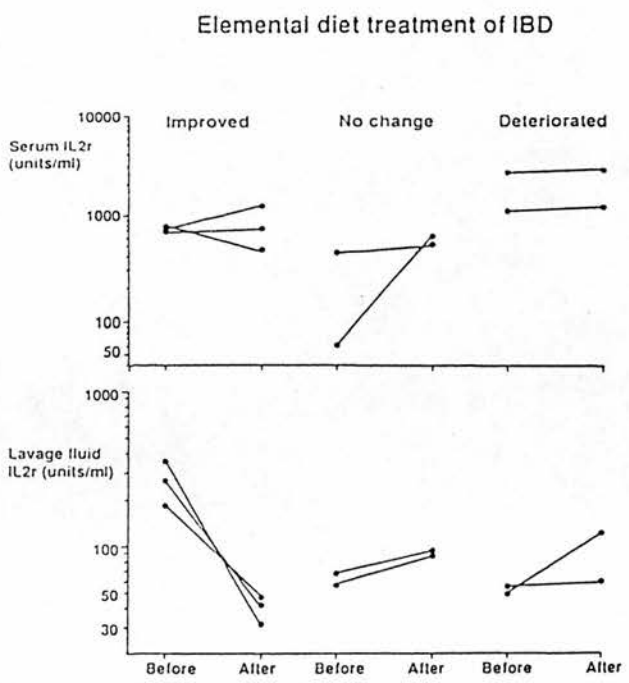

Fig. 3. Concentrations of sIL2R in serum and WGLF from patients with IBD before and at the end of a period of elemental diet treatment.

In the case of IBD, WGLF IgG content allows the separation of those with active and inactive disease. The results clearly show that whereas elastase content was low or absent in all but two of the patients with inactive IBD, those with unequivocally active disease clearly segregated into two groups, with either very high or absent neutrophil elastase. Preliminary analysis suggests that, at least in Crohn's disease, a high value for neutrophil elastase is associated with disease of the colon rather than the small bowel.

Cytokines

There is considerable interest in immunoregulatory and inflammatory cytokine networks within the gut mucosa. In preliminary studies we found that WGLF was unsuitable for bio-assays of cytokines, but that ELISA and radio immunoassay techniques could readily be applied.

As part of a programme of research on the immunological effects of treatment with elemental diet, studies of the marker of T cell activation, soluble IL2 receptor, have been carried out in a group of patients before and at the end of a 2–3 week period of treatment with the elemental diet E028. This was a heterogeneous group of patients, many of whom had failed other treatments. As shown in Fig. 3, the elemental diet treatment had no effect on serum levels of sIL2R, which were higher overall in the group of non-responders. However, the few patients who responded clinically to elemental diet treatment were characterized by an initially high level of sIL2R in WGLF, and the clinical response to

diet treatment was associated with a fall in this marker of gut T cell activation.

This is the first evidence of an immunological effect of elemental diet treatment in IBD: characterization of the pattern of immune activation in IBD patients may be a new way to identify patients likely to respond to novel forms of immunomodulatory intervention, and we are currently investigating this issue.

CONCLUSIONS

Imaging techniques, based on radioisotope labelled leukocytes, are invaluable in the assessment of pyogenic complications of IBD, and will also show the distribution of actively inflamed, ulcerated, macroscopically affected gut. The newly introduced technique of whole gut lavage provides a complementary approach to the analysis of inflammation and disease activity which is likely to be most valuable in the 5–10% of patients with complex or long-standing disease, and in the setting of clinical trials.

ACKNOWLEDGEMENTS

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EDITORIAL REVIEW

Mucosal immunology research design should address not just cytokines and cells, but also clinical data and controls

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(Accepted for publication 31 March 1994)

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic, incurable, disabling, inflammatory bowel diseases (IBD) which affect about 70 000 people in the UK. Although symptoms of the two conditions are similar, and they share some pathological and immunological features, they should be considered for research purposes as completely separate diseases of unknown and probably different etiologies. Disease activity and inflammation wax and wane unpredictably and, particularly in CD, indolent tissue destruction with fibrosis can cause bleeding, perforation, fistula formation and stricture—potentially life-threatening complications.

In clinical practice a range of techniques of clinical investigation are available, including several based on immunological approaches and reagents, to characterize the separate components of the patient's ill health. We recommend careful consideration of the distribution of grossly affected intestine, peri-anal disease, IBD disease activity, destructive ulceration, malnutrition, infection, side effects of drugs, gut losses of blood and protein, psychological factors, IBD-associated and independent diseases of other organs, and occasionally other, rarer problems. By this means, the clinician can devise appropriate management strategies for that particular patient at that time.

An appreciation of this clinical heterogeneity is also essential for the proper design of research studies in IBD, whether based on the sciences of epidemiology, nutrition, pharmacology, microbiology or immunology. Profound abnormalities of immunological status or the inflammatory response may be due to factors entirely unrelated to the primary disease process, such as global or single nutrient deficiency, immunomodulatory or cytotoxic drugs, or antibiotics. Account must also be taken of sex and age distribution of affected individuals, and the fact that by some means or other, material from healthy age- and sex-matched controls must be obtained to allow a proper interpretation of the findings in disease. Some cellular and molecular events are common to all ulcerative diseases, and some of the strikingly abnormal findings in IBD (e.g. high titres of serum and mucosal antibodies to bacterial and food antigens) may be merely epiphenomena, and occur in any situation where mucosal integrity is extensively breached. Thus, ideally, material should also be examined from patients with other ulcerative diseases of the gut, such as radiation colitis or infection.

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Many different approaches are being used in the search for the underlying causes of UC and CD, and to identify the presumed environmental factors which may trigger clinical relapse. Increasingly sophisticated techniques are becoming available to measure, both in healthy and diseased tissue, the precise immunological effects of immunosuppressive, cytotoxic, antibiotic and dietary treatments. These approaches may be useful for the identification of those patients likely to respond well to a particular second-line treatment—this would surely be cost-effective in the long run, in view of the expense of some drugs and of elemental diets.

In this difficult type of clinical research, material will become available for study in an often unpredictable way, particularly from the important group of newly presenting, untreated cases. We recommend that the collaborating clinician and immunologist agree, before the work begins, on a few broad subdivisions of the patient mix which will allow reasonably homogeneous groups, of adequate reasonable size, for proper interpretation and statistical analysis. There will be some patients who do not fit in to the pre-planned groupings. Their data should also be carefully examined. Unusual results, or a highly atypical case, may be the stimulus to entirely new avenues of hypothesis and investigation.

Neutrophils are the predominant effector cells within the active lesions of IBD and probably mediate much of the tissue damage. Indium^{111}-labelled leucocyte studies have demonstrated rapid accumulation of radiolabelled cells in the inflamed intestine, followed by migration into the crypts and thence into the intestinal lumen [1,2]. These studies showed that monitoring neutrophil migration could be of potential value in the clinical assessment of IBD patients. The introduction of ^{99m}Tc-HMPAO as a leucocyte label further refined the technique and provides much clearer scans [3].

There are several groups of molecule which may be acting as neutrophil chemoattractants in IBD. These include complement, eicosanoid and cytokine families and proinflammatory bacterial peptides. Recent studies have focused attention on IL-8, a non-glycosylated single peptide chain which mediates activation and chemotactic attraction of neutrophils [4]. The human neutrophil expresses at least two distinct receptors, high and low affinity, for IL-8 [5,6]. This cytokine was originally isolated from endotoxin-treated monocytes, but a variety of other cells may be induced to produce IL-8, and stimuli include IL-1 and tumour necrosis factor-alpha (TNF-α). Evidence that IL-8 may be implicated in the local tissue lesion of IBD comes from rectal and sigmoid perfusion studies, which show that

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myeloperoxidase released from neutrophil granules correlates with IL-8 in the perfusate in UC [7].

In an elegantly simple study reported in this issue, Mitsuyama *et al.* [8] demonstrate that raised IL-8 content of affected colonic mucosa in active IBD correlates with neutrophil counts in the mucosa. This is strong evidence of a central role for IL-8 in the pathogenesis of neutrophil infiltration. These workers also observed that mucosal IL-8 levels in macroscopically unaffected areas of the colon were similar to those in colonic biopsies from immunologically normal control patients. Measurement of serum IL-8 concentration was unhelpful. The study design conformed to the criteria set out above. The well described patient groups included a reasonable number of untreated cases, and also patients with ischaemic and infective colitis (several of whom had detectable IL-8, although at lower levels than in the active IBD patients). It is important to keep in mind, when comparing these data with those in other reports, that their patients and biopsies were designated as active or inactive on the basis of gross appearance of the colonic mucosa at endoscopy. We, using the new technique of whole gut lavage [9], and others, using a range of clinical tests [10], have shown that inflammatory 'disease activity' is a separate phenomenon from mucosal ulceration and tissue destruction.

An important unanswered question is the source of IL-8 in IBD. Mucosal monocytes and macrophages are known to produce IL-8, and IL-8 is also expressed by human epithelial cell lines [11]. There may be bidirectional communication between intestinal epithelial cells and mucosal immune cells, whereby IL-1 and TNF-α released by activated macrophages may result in increased IL-8 secretion by intestinal epithelial cells. However, caution is needed in extrapolating data from *in vitro* studies—epithelial cell lines also express IL-1 and yet immunohistochemical and *in situ* hybridization studies have shown that lamina propria mononuclear cells and not epithelial cells are the source of IL-1 in intestinal mucosal biopsies [12].

One potential mechanism of action of IL-8 is via regulation of expression of leucocyte adhesion molecules. Binding of neutrophils to the vascular endothelial cells may be mediated through well characterized adhesion molecules such as CD11a/CD18 complex (LFA-1) which binds to CD54 (ICAM-1). In active IBD, the expression of CD54 is strikingly increased [13]. Another family of adhesion molecules, the LEC-CAM, present on neutrophils and endothelium, mediate adhesion by a lectin domain. This promises to be one of the central areas for future research and therapeutic intervention.

Some of the traditionally investigated chemoattractants such as leucotriene-B_4 may be a secondary consequence of neutrophil infiltration, while other mediators such as those of the complement family (C3b, C5a) may be important in the final pathway of tissue injury by perpetuating the inflammation. Though several groups have investigated the role of platelet-activating factor (PAF) in IBD, no correlation between degree of tissue inflammation or disease activity and PAF has been reported. An important area that needs further experimental clarification is the relationship of neutrophil chemoattractants of bacterial origin such as formyl-methinyl-leucyl-phenylalanine (FMLP) and production of tissue mediators such as IL-8.

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Investigation of neutrophil migration into the gut by cytology of whole gut lavage fluid

Louise M. Handy, Subrata Ghosh and Anne Ferguson

Objective: Radiolabelling studies have shown that neutrophils migrate into the gut lumen in inflammatory intestinal diseases. We aimed to establish whether whole gut lavage fluid can be used to investigate intestinal neutrophilia.

Design: A prospective single centre study comparing inflammatory bowel disease patients, patients with other gastrointestinal diseases and normal controls.

Methods: Gut lavage with a polyethylene glycol-based solution was performed in 56 patients (29 with inflammatory bowel disease). Gut lavage fluid samples were collected when the rectal effluent was clear. Cells were then separated by density gradient centrifugation, counted, and cytospin preparations examined.

Results: High cell counts ($> 3.7 \times 10^5$/ml), predominantly neutrophils, were present in 12 out of 29 inflammatory bowel disease patients and in two out of 27 others. High cell counts and luminal neutrophilia characterized active ulcerative colitis and active colonic Crohn's disease, but not active small bowel Crohn's disease, or inactive disease.

Conclusion: Whole gut lavage fluid samples can be used to investigate luminal inflammatory cells. We observed previously unrecognized differences between small bowel and colonic Crohn's disease using this method. The mechanisms of neutrophil migration into the gut lumen may differ with disease distribution.

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Keywords: Whole gut lavage, gut neutrophils, intestinal inflammation, Crohn's disease, ulcerative colitis

Introduction

Whole gut lavage is a relatively new technique for the non-invasive investigation of gut immunity [1,2]. It can be used to examine mucosal immunoglobulins and antibodies, to measure gut losses of blood and protein, and for studies of local immunoregulatory and pro-inflammatory cytokines. It has been known for several years that tumour cells can be identified by the cytological examination of whole gut lavage fluid (WGLF) in patients with gastrointestinal cancers [3–6].

Experiments with intravenously administered mixed leukocyte preparations, labelled with ^{111}In or ^{99m}Tc, have shown that tumour cells rapidly localize in the inflamed bowel, migrate into the bowel lumen, and are excreted in faeces [7,8]. Quantitation of the faecal excretion of these radiolabelled cells has been used to assess disease activity in patients with inflammatory bowel disease (IBD) [9–11].

It appeared probable that leukocytes in the gut lumen would also be detected in WGLF and, if so, a simple, non-isotopic method to examine this aspect of gut inflammation could be developed. Therefore, we collected, counted, and examined microscopically the cells in WGLF from a series of well characterized patients with IBD and other conditions in order to assess how luminal neutrophilia relates to the type and distribution of IBD, and to disease activity.

The study protocol was approved by the Medicine Subcommittee of the Lothians Area Ethics of Research Committee.

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Materials and methods

Subjects

WGLF samples from 56 patients were used for cytological assessment. Each patient underwent gastrointestinal assessment with appropriate combinations of upper endoscopy, colonscopy, jejunal biopsy, other histology and contrast radiology. Laboratory investigations included determinations of haemoglobin, white cell count, erythrocyte sedimentation rate, albumin and C-reactive protein. All medication and diet at the time of lavage were recorded.

Thirteen patients were immunologically normal, with anatomically normal gastrointestinal tracts, and were used as controls. Twenty-nine patients had IBD: 12 had active Crohn's disease (five with colonic Crohn's disease including additional small bowel involvement; four with small bowel Crohn's disease; three with other, for example, perianal Crohn's disease); 10 had inactive Crohn's disease (five with colonic Crohn's disease, two with small bowel Crohn's disease, three with other Crohn's disease); three had active ulcerative colitis; and four had ulcerative colitis in remission. Of the ulcerative colitis patients, four had pancolitis, one had distal colitis and two had proctitis. IBD was classified as active when the WGLF immunoglobulin (Ig) G concentration was greater than 10 μg/ml. Six patients had other benign, upper gastrointestinal tract diseases, and seven had colonic diseases other than IBD (two with radiation colitis, two with benign polyps, and one each with infectious colitis, diverticulitis and stercoral ulcer). An eighth patient, with colon carcinoma complicating longstanding inactive ulcerative colitis, was classified as belonging to this 'miscellaneous colonic disease' group.

Lavage protocol

The patients drank an isotonic polyethylene glycol-based fluid (Klean-prep, Norgine Ltd, Oxford, UK), supervised by an experienced nurse, as described elsewhere [12]. The first completely clear fluid specimen passed per rectum was collected for analysis. Cytological assessment was performed immediately after collection as described below. Aliquots of the specimen were also filtered, processed by the addition of protease inhibitors, stored at −70°C and later assayed for IgG by enzyme-linked immunosorbent assay [12].

Disease activity in IBD patients

One of the advantages of using WGLF for IBD research is that disease activity can be graded by measuring IgG concentration in WGLF. This has been validated by Choudari *et al.* [12] who found that the Crohn's disease activity index (CDAI) for Crohn's disease and the Powell Tuck index (PTI) for ulcerative colitis correlated well with WGLF IgG concentration. In the same series of patients, an experienced physician's global assessment also correlated with the CDAI or PTI, and with the WGLF IgG concentration [13].

For the purposes of statistical analysis, IBD patients were classified as active when the IgG concentration in WGLF was greater than 10 μg/ml, and inactive when the IgG level was 10 μg/ml or less. In this study, we also compared WGLF IgG concentration values in the 29 IBD patients with a physician's clinical opinion on whether the disease was active or inactive (based on symptoms, clinical signs and the results of haematological and biochemical blood tests). The categorization as active or inactive, based on IgG concentration, was the same as the clinical classification in all patients. The conventional laboratory indices of disease activity in the IBD patients (serum albumin, C-reactive protein, white cell count and erythrocyte sedimentation rate) and the physician's clinical classification into active or inactive disease are given in Table 1.

Cytology preparations

Each WGLF specimen (25 ml) was centrifuged at 850 **g** for 10 min. The supernatant was discarded and the pellet resuspended in 5 ml Hanks' balanced salt solution (Sigma Chemical Co. Ltd, Poole, UK). This was layered on top of a discontinuous density gradient using

Table 1. Conventional laboratory markers of disease activity in 29 inflammatory bowel disease (IBD) patients and physician's global assessment.

	Median (range)				
IBD group	Serum albumin (g/l)	C-reactive protein (mg/dl)	White cell count (×10⁹/l)	ESR (mm/1st h)	No. patients classified as having active disease by physician
Active CD					
Colonic (n = 5)	37 (35–42)	7.6 (< 1.5–16.5)	11.6 (9.5–14.5)	46 (20–89)	5
Small bowel (n = 4)	38 (23–41)	< 1.5	8.5 (6.0–10.2)	34 (7–74)	4
Others (n = 3)	40 (22–43)	1.5 (< 1.5–2.0)	7.3 (5.4–7.8)	27 (4–44)	3
Inactive CD					
Colonic (n = 5)	42 (34–45)	< 1.5	8.7 (6.5–15.9)	2 (1–13)	0
Small bowel (n = 2)	38, 41	< 1.5	5.1, 9.4	4, 12	0
Others (n = 3)	42 (40–45)	< 1.5	5.8 (5.5–6.2)	27 (2–100)	0
Active UC (n = 3)	39 (39–43)	< 1.5	8.4 (7.5–9.3)	13 (2–24)	3
Inactive UC (n = 4)	45 (41–46)	< 1.5	6.6 (5.4–7.2)	12 (6–40)	0

ESR, erythrocyte sedimentation rate; CD, Crohn's disease; UC, ulcerative colitis.

Histopaque 1119 and 1077 (Sigma). After centrifugation for 30 min at 700 **g**, the two resultant layers were harvested separately. The suspensions were washed with Hanks' balanced salt solution and the pellets resuspended in 1 ml. Cell counts were performed with a haemocytometer, and slides of the two suspensions were made using a Cytospin cytocentrifuge (Shandon Southern Products Ltd, Runcorn, UK) operated for 10 min at 90 **g**. The slides were air-dried, fixed in methanol and then stained by the May–Grunwald–Giemsa protocol. Slides were examined with an Ortholux II microscope (Leitz, Wetzlar, Germany) and the cell types present recorded. In addition, we graded the number of neutrophils in the preparation subjectively as few or none, some or many. Slides were immunostained using monoclonal antibodies for anti-CD3 (Scottish Antibody Production Unit, Carluke, UK), anti-L26 (DAKO Ltd, High Wycombe, UK) and anti-CD68,KPI (DAKO) to detect T lymphocytes, B lymphocytes and macrophages. The three-stage streptavidin biotin alkaline phosphatase technique was used [14].

Statistical analysis

Comparisons between the different groups of subjects were performed by the Mann–Whitney test and Chi-squared test with Yates correction. P values < 0.05 were considered significant.

Results

WGLF cytology

In our first cytological examinations of WGLF, we used either samples filtered through two layers of surgical gauze before centrifugation, counting and cytocentrifugation, or samples centrifuged and layered onto one high density separation medium only. These methods produced slide preparations which contained considerable amounts of debris and many bacteria, in addition to variable numbers of cells. A modification of the technique using two density separation media (described in Methods) greatly reduced bacterial contamination and debris. We had anticipated that neutrophils and lymphocytes would layer separately at the two interfaces during gradient centrifugation, but this did not occur. Most cells were recovered from the upper of the two layers, and the cell types seen in the top layer were also seen, although in fewer numbers, in the bottom layer. The data reported below are the total cell counts for both layers added together, and cytological descriptions for both layers combined.

The cells observed were neutrophils, squamous cells, enterocytes, eosinophils, macrophages, erythrocytes, and, very occasionally, lymphocytes; cells from a patient with active Crohn's disease are shown in Fig. 1. Some samples contained yeasts, and bacteria were ubiquitous.

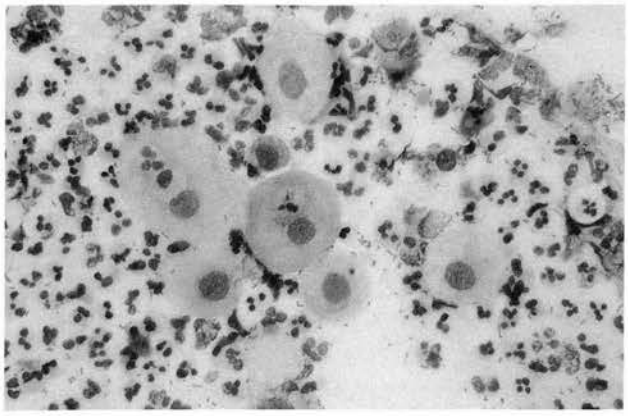

Fig. 1. Typical presentation of whole gut lavage fluid (WGLF) cytology from a Crohn's disease patient, showing neutrophils, squamous cells, a macrophage, and some degenerate enterocytes. (Magnification ×40; May–Grunwald–Giemsa stain).

Cell counts

Figure 2 shows the cell counts for the different disease groups. Specimens with many neutrophils seen on cytospin slides are highlighted. In normal controls, the mean + 2 SD of the cell count was 3.7×10^5/ml WGLF, and this value was taken as the upper limit of the reference range. In the one control with a cell count above this level, microscopy showed that these were predominantly squamous cells. Nine of the 22 patients with Crohn's disease and three of the seven with ulcerative colitis had high cell counts, as did one of the 14 patients with other disease (cell count 3.8×10^5/ml in WGLF from a patient with infective colitis). Information on the cell types in WGLF from IBD patients are given below. Two of the 14 patients with other disease had many neutrophils in cytospins of WGLF (one patient with infective proctitis who also had high WGLF IgG and a patient with colonic carcinoma and longstanding inactive ulcerative colitis).

Cell counts in IBD according to disease activity and anatomy of involvement

Figure 3 shows the cell counts in WGLF from IBD patients subdivided into ulcerative colitis, colonic Crohn's disease, small bowel Crohn's disease and Crohn's disease with other distribution. Results are presented in relation to WGLF IgG concentrations, the objective measure of disease activity. Table 2 summarizes the values for selected subgroups of patients together with the results of statistical analyses. In general, patients with active IBD had higher cell counts than those with inactive disease ($P < 0.05$). Despite the relatively small number of patients studied, the differences in cell number were significant for active IBD compared with normal controls ($P < 0.05$). However, the subgroup with active small bowel Crohn's disease did not conform; cell counts were

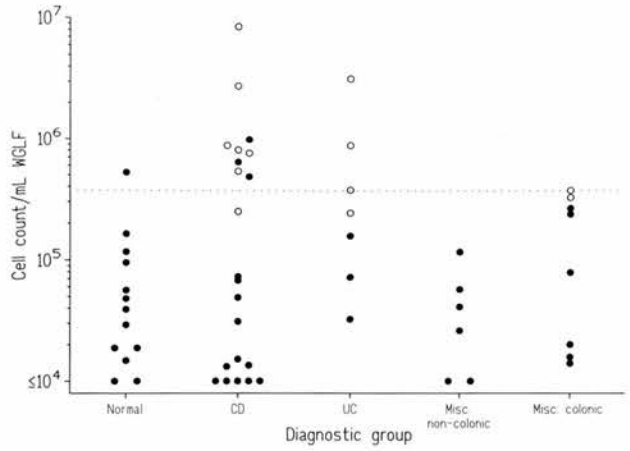

Fig. 2. Number of cells/ml whole gut lavage fluid (WGLF) plotted against diagnostic group, highlighting subjects with many neutrophils. (○) many neutrophils seen; (●) all other subjects; (.....) the count/ml equal to the mean + 2 SD of the normal controls.

in the normal range in three of the four patients studied (Fig. 3).

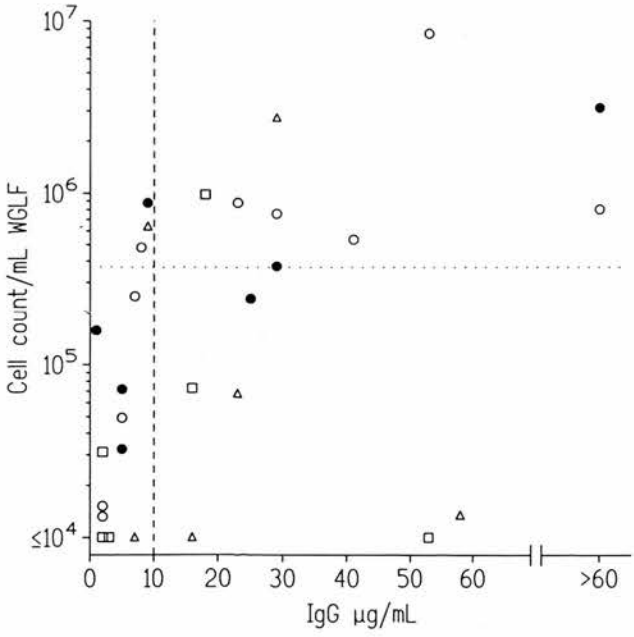

Fig. 3. Cell count/ml whole gut lavage fluid (WGLF) plotted against WGLF immunoglobulin (Ig) G concentration for patients with inflammatory bowel disease (IBD). (○) Colonic Crohn's disease; (△) small bowel Crohn's disease; (□) other Crohn's disease; (●) ulcerative colitis; (.....) the count/ml equal to the mean + 2 SD of the normal controls and (- - - -) the upper limit of normal for IgG concentration.

Cell types in IBD according to disease activity and anatomy of involvement

Cytospin preparations showed many neutrophils in six of the 10 specimens from colonic Crohn's disease patients

Table 2. Cell counts in whole gut lavage fluid in inflammatory bowel disease (IBD) groups and controls.

Diagnostic group	n	Cell count/ml [median (range)]	*P*
Controls	13	3.9×10^4 (1×10^4–5.2×10^5)	
Active IBD	15	5.4×10^5 ($<1\times10^4$–8.4×10^6)	<0.05*
Inactive IBD	14	4.1×10^4 ($<1\times10^4$–8.8×10^5)	<0.05†
Active CD	12	6.5×10^5 ($<1\times10^4$–8.4×10^6)	NS*
Inactive CD	10	2.3×10^4 ($<1\times10^4$–6.4×10^5)	<0.05‡
Active CD			
Colonic	5	8.1×10^5 (5.4×10^5–8.4×10^6	<0.01*
Small bowel	4	4.1×10^4 ($<1\times10^4$–2.7×10^6)	NS*
Other	3	7.3×10^4 ($<1\times10^4$–9.8×10^5)	NS*
Inactive CD			
Colonic	5	4.9×10^4 (1.3×10^4–4.8×10^5)	NS*
Small bowel	2	$<1\times10^4$, 6.5×10^5	
Other	3	$<1\times10^4$ ($<1\times10^4$–3.1×10^4)	NS*
Active UC	3	3.8×10^5 (2.4×10^5–3.1×10^6)	<0.05*
Inactive UC	4	1.2×10^5 (3.2×10^4–8.8×10^5)	NS§

*Versus normal controls; †versus patients with active IBD; ‡versus patients with active Crohn's disease (CD); §versus patients with active ulcerative colitis (UC). NS, not significant.

($P<0.01$ compared with the controls) from one of the 12 other Crohn's disease patients (not significant) and from four of the seven samples from ulcerative colitis patients ($P<0.02$). When these data were examined in relation to disease activity, the importance of the site of IBD became clear. Many neutrophils were seen in cytospin preparations from the three patients with active ulcerative colitis, the five with colonic Crohn's disease, but in only one of the four patients with active small bowel Crohn's disease (a patient with ileal disease complicated by an abscess who was also receiving high doses of non-steroidal anti-inflammatory drugs for ankylosing spondylitis) (Table 3). There was no relationship between disease distribution and the number of neutrophils in cytospin preparations from patients with inactive IBD.

Other cell types

Eosinophils were seen in preparations from three of the 22 patients with Crohn's disease, three of the seven ulcerative colitis patients and in the patient with colonic carcinoma complicating ulcerative colitis; in all of these, neutrophils were also present in the WGLF. Enterocytes and squamous cells were frequently seen in slides both with and without neutrophils. Erythrocytes were found occasionally in slides from IBD patients, and macrophages were seen occasionally in a variety of clinical conditions. Lymphocytes were rarely observed and occurred in low

Table 3. Presence of neutrophils in different diagnostic groups.

Diagnostic group	n	None/few	Some	Many
Normal	13	13	0	0
Active IBD				
Colonic CD	5	0	0	5
Small bowel CD	4	3	0	1*
Other CD	3	2	1	0
UC	3	0	0	3
Inactive IBD				
Colonic CD	5	3	1	1
Small bowel CD	2	2	0	0
Other CD	3	3	0	0
UC	4	2	1	1
Other non-colonic disease	6	5	1	0
Other colonic disease	8	5	1†	2‡

*Complicated by abscess and long-term high dose non-steroidal anti-inflammatory drugs for ankylosing spondylitis; †diverticulitis; ‡infective colitis and colon carcinoma complicating ulcerative colitis (UC). IBD, inflammatory bowel disease; CD, Crohn's disease.

numbers, mostly in patients with IBD. Immunostaining of the few specimens containing lymphocytes confirmed the presence of B cells, plasma cells, and T cells, but the general paucity of lymphocytes was striking. No malignant cells were seen.

Discussion

Many approaches are being used to study gut inflammation and its pathogenesis. These include immunocytochemical investigations of biopsies and resected specimens, radiolabelled leukocyte studies, perfusion of isolated loops of small bowel or colon with measurements of proinflammatory mediators, and various immunological tests on faecal extracts [9–11,15]. Radiolabelled leukocyte tests have provided most of the recent descriptive information on the migration of cells into the bowel wall and then into the gut lumen, but more direct methods are needed to investigate the mediators and chemotactic agents involved.

The use of whole gut lavage for research on intestinal antibodies has been described by Gaspari *et al.* [16]. We have simplified and standardized the clinical protocol to create a whole gut perfusion system, relatively unaffected by disease, age, drugs or diet (Brian and Ferguson, submitted for publication). After appropriate specimen processing, WGLF analysis can be used to investigate gut immunoglobulins and antibodies [17], to detect plasma protein leak [18] and bleeding into the gut [19], and for studies of mucosal cytokines [13], with minimal degradation of the cells and molecules of interest.

We have now shown that peroral whole gut lavage can also be used to investigate inflammatory cells within the intestinal lumen. Studies of cells and their products can be combined with biochemical and immunological assays on WGLF to provide detailed analysis and characterization of immunological and inflammatory events in the gut. The capacity to investigate the relatively inaccessible small bowel is clearly advantageous, and for research in IBD, the measurement of IgG concentration in WGLF provides a simple and objective measure of disease activity. A heterogeneous group of IBD patients can thus be subdivided and stratified into subsets with comparable disease activity to allow investigation of other factors such as corticosteroid or diet treatments, or disease distribution [15]. By applying WGLF analysis in other forms of gastrointestinal disease, we can also determine whether a particular immunological phenomenon is truly a feature of ulcerative colitis or Crohn's disease, or is simply a non-specific expression of tissue injury in the gut.

It is not surprising that inflammatory cells have been detected in WGLF samples, since it is already known that smears from centrifuged whole gut lavage fluid provide adequate specimens for cytological diagnosis of colonic carcinoma [20] and that neutrophils can be found in the faeces of IBD patients [9]. Using the method described, gut luminal cells can be counted and characterized by cytology. Our results show high counts of luminal cells, mainly neutrophils, in most patients with active IBD. However, it appears that this luminal neutrophilia is not a feature of small bowel Crohn's disease. Only one of our patients with ileal Crohn's disease had a high WGLF cell count and there were two associated complicating factors (NSAID intake and an abscess communicating with the gut).

Only one study has reported that faecal excretion of radiolabelled leukocytes is much higher in colonic than in small bowel Crohn's disease [21]; in other reports, regional data are either not presented [10,22], or there was no observed effect of regional distribution [23]. Most of these reports include small numbers of patients, as in this study on WGLF cytology. WGLF cytology must be performed on fresh samples and is time consuming, but we have found recently that the WGLF concentration of granulocyte elastase is a useful marker of neutrophils and correlates well with cell count. By using this assay in large numbers of specimens, we have confirmed a striking effect of disease distribution on luminal neutrophils. (unpublished data).

Eosinophils, although detected less frequently in WGLF than neutrophils, were observed in WGLF from six of the 15 patients with active IBD and from one patient with carcinoma complicating ulcerative colitis. In all of these patients there were also many neutrophils in the WGLF specimens. Preliminary work on the assay of a biochemical marker for eosinophils, eosinophil cationic protein, supports this finding (unpublished data).

Further studies with WGLF may elucidate the stimuli for inflammatory cell migration into the gut, and local activation. Various substances may act as chemoattractants, including interleukin-8, bacterial peptides such as formyl-methionyl-leucyl-phenylalanine, leukotriene-B4, complement factors such as C5a, and platelet-acti-

vating factor [15], and it should be possible to study these in parallel with the objective measures of disease severity which we have developed already.

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Investigation of Neutrophils in the Gut Lumen by Assay of Granulocyte Elastase in Whole-Gut Lavage Fluid

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Handy LM, Ghosh S, Ferguson A. Investigation of neutrophils in the gut lumen by assay of granulocyte elastase in whole-gut lavage fluid. Scand J Gastroenterol 1996;31:700–705.

Background: Intestinal neutrophils can be studied by radiolabelling techniques and by cytology of whole-gut lavage fluid. Our aim was to evaluate the use of a biochemical test for the presence of these cells in whole-gut lavage fluid. *Method:* Whole-gut lavage was performed by having the patients drink a polyethylene-glycol-electrolyte solution; the clear fluid passed per rectum after complete bowel cleansing had been collected. In 203 patients granulocyte elastase was assayed in sonicated unfiltered lavage fluid, using the specific enzyme substrate L-pyroglutamyl-L-prolyl-L-valine-*p*-nitroanilide. Free granulocyte elastase was also assayed in filtered (that is, cell-free) lavage fluid in 39 of the 43 patients in whom the enzyme was present in unfiltered fluid. In 47 of the patients, cells were also separated by density gradient centrifugation, and counted. *Results:* Granulocyte elastase concentration correlated significantly with cell count ($r = 0.80$, $p < 0.001$). Granulocyte elastase concentration was high (>100 nkat/l) in fluid from 25 of 68 inflammatory bowel disease patients and 6 of 135 others with radiation colitis, diverticulitis, pericolic abscess, and use of non-steroidal anti-inflammatory drugs. In patients with detectable total granulocyte elastase, cell-free granulocyte elastase was present in 11 of 29 with inflammatory bowel disease and 1 of 10 others. *Conclusion:* Whole-gut lavage fluid samples can readily be used to investigate luminal inflammatory cells.

Key words: Granulocyte elastase; intestinal neutrophils; whole-gut lavage

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Whole gut lavage is a relatively new technique for non-invasive investigation of gut immunity (1, 2) and can be used for research on mucosal immunoglobulins and antibodies, to measure gut losses of blood and protein, and for studies of local immunoregulatory and pro-inflammatory cytokines. Cytologic investigation of whole-gut lavage fluid (WGLF) has shown tumour cells in some patients with gastrointestinal cancers (3–5), and in a study of 56 patients we have recently observed the presence of many neutrophils in WGLF from patients with active ulcerative colitis (UC) or colonic Crohn's disease (CD) (6).

The granules of polymorphonuclear leucocytes contain enzymes such as elastase (7) and myeloperoxidase (8). Since granulocyte elastase (GE) can be detected in faeces from patients with gut inflammation (9, 10), it seemed likely that this biochemical assay could also be used for the investigation and quantification of intestinal granulocytes in WGLF.

The aim of this study was to explore whether GE assay in WGLF may be used to study neutrophil migration into the lumen of the gut in inflammatory conditions.

MATERIALS AND METHODS

Subjects

We studied WGLF samples from 203 patients in whom the gastrointestinal tract was assessed by an appropriate combination of upper endoscopy, colonoscopy, jejunal biopsy, other histology and contrast radiology. Inflammatory bowel disease (IBD) was classified as clinically active when the WGLF IgG concentration was greater than 10 μg/ml as validated previously by our group (11, 12). In the present study we have again compared values for WGLF IgG concentration in the 68 IBD patients with a physician's overall clinical decision as to whether the disease was active or inactive (on the basis of symptoms, clinical signs, and results of haematologic and biochemical blood tests). The categorization as active or inactive on the basis of IgG concentration (>10 μg/ml or ≤10 μg/ml) was the same as the clinical classification in 65 of the 68 cases. Of the three with discrepant results, two had severe UC and were receiving intensive medical treatment with intravenous steroids; they were clinically graded as having active disease, whereas WGLF IgG levels were 9 and 6 μg/ml. The third patient had colonic CD, was taking maintenance oral steroid therapy, had few symptoms and normal inflammatory values in blood apart from an increased erythrocyte sedimentation rate. She was clinically graded as inactive, but her WGLF IgG concentration was 16 μg/ml. For the purposes of analysis and statistical comparisons in this report, we have used the objective measure, WGLF IgG concentration: IBD active when IgG > 10 μg/ml and inactive when IgG ⩽ 10 μg/ml.

Thirty-one patients had a normal gastrointestinal tract and

Table I. Detection of granulocyte elastase (GE) in inflammatory bowel disease patients and normal subjects

Diagnostic groups*	*n*	GE present	*P* versus normals	*P* versus other relevant groups	WGLF GE conc. (nkat/l) median, range	*P* versus normals	*P* versus other relevant groups
Normals	31	4			<39, <39–113		
Active CD	23	14	<0.01		300, <39–1435	<0.001	
Inactive CD	20	5	NS	<0.05 versus active CD	<39, <39–571	NS	<0.005 versus active CD
Active UC	10	6	<0.01		103, <39–1550	<0.001	
Inactive UC	15	6	NS	NS versus active UC	<39, <39–370	NS	NS versus active UC

* CD = Crohn's disease; UC = ulcerative colitis.

acted as controls. Forty-three patients had CD, and 25 had UC. Sixty-three patients had miscellaneous non-colonic gastrointestinal diseases, and 41 had colonic diseases other than IBD.

Lavage protocol

The patients drank an isotonic polyethylene glycol-based fluid (Klean-prep, Norgine Ltd., Headington, Oxford, UK), supervised by an experienced nurse, as described elsewhere (11). The first completely clear fluid specimen passed per rectum was collected for analysis. Aliquots of unfiltered, unprocessed specimens were stored at −70 °C until assayed for GE. The rest of the specimen was filtered through glass microfibre filters (Whatman GF/A, Whatman International Ltd, Maidstone, England) with a pore size of 1.6 μm, and aliquots were stored without further processing for assay of free GE. Other aliquots of the filtered specimen were processed by the addition of protease inhibitors as previously described (11), stored at −70 °C, and later assayed for IgG by enzyme-linked immunosorbent assay (ELISA) (1).

In 47 of the above patients cytologic assessment was performed on the first clear specimen immediately after collection as previously described (6).

The protocol was approved by the Medicine Subcommittee of the Lothians Area Ethics of Research Committee.

Cytology preparations

A specimen of unfiltered, unprocessed WGLF (25 ml) was centrifuged immediately after collection at 850 *g* for 10 min. The supernatant was discarded, and the pellet resuspended in 5 ml Hanks' balanced salt solution (Sigma Chemical Co. Ltd, Poole, UK). This was layered on top of a discontinuous density gradient using Histopaque 1119 and 1077 (Sigma). After centrifugation for 30 min at 700 *g* the two resultant layers were harvested separately. The suspensions were washed with Hanks' balanced salt solution and the pellets resuspended in 1 ml. Cell counts were performed with a haemocytometer.

Granulocyte elastase assay

Total GE. The GE assay technique is based on the method used for measurement of elastase in granulocyte extracts from whole blood. The highly specific substrate for GE, L-pyroglutamyl-L-prolyl-L-valine-*p*-nitroanilide (13), was obtained from Quadratech (Epsom, UK) and was dissolved in dimethylsulphoxide and diluted four times in water to give a final concentration of 2 mmol/l. Approximately 1 μl of Triton X-100 (Sigma) was mixed into 500 μl of thawed, unfiltered, unprocessed WGLF (the precise amount of Triton is not critical (13)). Each sample was sonicated at 0 °C for 1 min three times, using a Rapidis A180G sonicator (Ultrasonics Ltd., Shipley, UK) to disrupt the cells present and release the enzymes contained in the granules. Two hundred microlitres of sample was added to 200 μl of buffer (Tris, 0.1 mol/l; NaCl, 0.96 mol/l; pH 8.3), and the mixture was incubated at 37 °C. Two hundred microlitres of substrate at 37 °C was added, and after exactly 3 min the reaction was stopped with 200 μl of 20% acetic acid. Blank tubes were run for all samples in the same manner but with the acetic acid added before the substrate. Absorbance was measured at 405 nm, and GE concentration calculated using the formula provided by Quadratech: (test A_{405} − blank A_{405}) × 2310 nkat/l. A crude human granulocyte extract was prepared by a modification of the method of Kramps et al. (14) and was included as a standard. The limit of detection of the assay was a GE concentration of 39 nkat/l. The between-assay coefficient of variation was 6%. The possibility of interference by the high concentration of polyethylene glycol in WGLF was excluded by comparing values for blood granulocyte suspensions in balanced buffer and in surrogate WGLF preparations; no difference was detected.

Free GE. Thirty-nine patients who had total GE detectable by the above assay also had GE measured in a filtered, unprocessed sample. The method described above was used without the sonication step. The process of filtration removed the granulocytes, and hence any GE detectable in this specimen should represent free or non-particulate GE.

Statistical analysis

Correlation coefficients were calculated using the Spearman rank correlation test. All 47 patients who had both cytology and GE estimations were included in the calculation of correlation coefficient, irrespective of whether they had detectable GE. Comparisons between different groups of

Table II. Non-inflammatory bowel disease patients with markedly increased granulocyte elastase (GE) levels (>100 nkat/l) in whole-gut lavage fluid

Diagnosis	IgG, μg/ml	GE, nkat/l	Comments*
Acute radiation colitis	236	2285	Radiotherapy for carcinoma cervix
Diverticulitis	27	132	Settled with antibiotics
Diverticular disease	3	240	On NSAIDs
Carcinoma colon	44	109	Caecal tumour with pericolic abscess
Systemic lupus erythematosus	1	210	On NSAID and prednisolone, 10 mg/day
Volunteer	1	113	Normal volunteer; repeat lavage after 3 weeks did not detect GE

* NSAID = non-steroidal anti-inflammatory drugs.

subjects were carried out using the Mann–Whitney test and the chi-square test with Yates correction. P values <0.05 were considered significant.

RESULTS

GE concentrations in WGLF

Results of GE assays in the 203 WGLF samples are summarized in Table I. In specimens collected from the 31 immunologically normal patients and volunteers, GE was detectable (≥39 nkat/l) in only 4 samples (values of 60, 74, 74, and 113 nkat/l). Results in the various patient groups were analysed and are presented as absolute GE values and as the number of patients with any detectable GE. Values >100 nkat/l WGLF are considered abnormally high.

Detection of GE was significantly more frequent than in normals, and absolute values were also significantly higher, for specimens from CD patients (19 of 43, $p < 0.01$; median <39, range <39–1435 nkat/l, $p < 0.001$) and from UC patients (12 of 25, $p < 0.01$; median <39, range <39–1550 nkat/l, $p < 0.001$). Only 5 of 41 patients with other colonic diseases and 3 of 63 patients with other non-colonic diseases had detectable GE. Neither these frequencies nor the absolute values differed significantly from those for the normal group.

One subject classified as normal and five with diseases other than IBD had high GE concentrations (>100 nkat/l). These patients are described in Table II. Three patients had clinical disorders compatible with exudation of neutrophils into the lumen—acute radiation colitis, diverticulitis, pericolic abscess communicating with the lumen; in the other two it seems likely that the explanation was non-steroidal anti-inflammatory drug (NSAID)-associated enteropathy. The normal subject was a volunteer in a study investigating mucosal immunity in smokers; in a further specimen of WGLF collected 3 weeks later GE was undetectable.

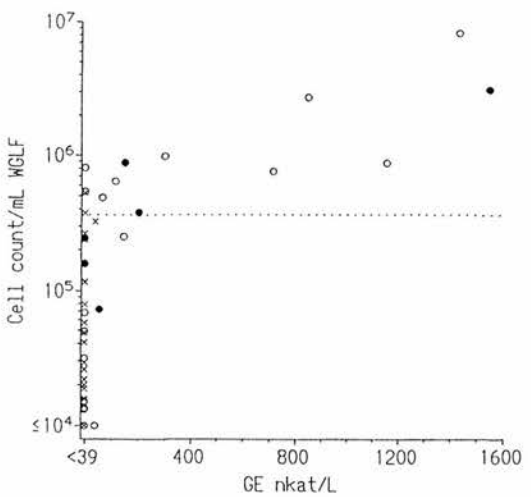

Fig. 1. Cell count per millilitre whole-gut lavage fluid (WGLF) plotted against granulocyte elastase (GE) concentration in WGLF. (○) = Crohn's disease; (●) = ulcerative colitis; (×) = all other diagnoses. The dotted line shows the count per millilitre equal to the mean + 2SD of the normal group.

Relationship between cell count and GE concentration in WGLF

Fig. 1 shows WGLF cell counts and total GE concentrations in the 47 patients studied by both methods. The correlations for the whole series, for CD, and for UC patients are all highly significant (all cases, $r = 0.80$, $p < 0.001$; CD, $r = 0.79$, $p < 0.001$; UC, $r = 0.98$, $p = 0.001$). There were 33 specimens in which GE was less than the 39 nkat/l detection limit.

Total and free GE

Aliquots of WGLF that had been filtered but not treated in

Table III. Detection of free granulocyte elastase (GE) in 39 patients with detectable total GE

Diagnostic groups*	No. with detectable total GE	No. with detectable free GE
Normals	3	0
CD	18	7
UC	11	4
Miscell. upper GI	2	0
Miscell. colonic	5	1†

* CD = Crohn's disease; UC = ulcerative colitis; GI = gastrointestinal.

† Radiation colitis.

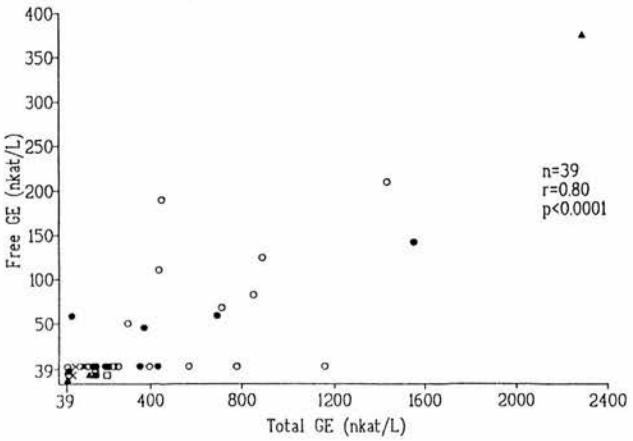

Fig. 2. The relationship between free and total granulocyte elastase (GE) in 39 patients with detectable total GE. (×) = Normals (n = 3); (○) = Crohn's disease (n = 18); (●) = ulcerative colitis (n = 11); (□) = miscellaneous upper GI (n = 2); (▲) = miscellaneous colonic (n = 5).

any other way were available for 39 of the 43 specimens in which GE had been detected in unfiltered specimens. Results of GE assays in these paired samples are summarized in Table III, and Fig. 2 shows the relationship between total and free GE. There was a significant correlation between free GE and total GE (r = 0.8; $p < 0.0001$). Eleven of 29 IBD patients with detectable total GE had detectable free GE, whereas none of the 3 normals and only 1 of the 7 diseased controls (the patient with radiation colitis) had detectable free GE. In the whole group the concentration of total GE in those with detectable free GE (median, 703 nkat/l; range, 55–2285 nkat/l) was significantly greater ($p < 0.001$) than that in those with undetectable free GE (median, 159 nkat/l; range, 39–1157 nkat/l). This was also true for CD patients in whom the concentration of total GE in those with detectable free GE (median, 714 nkat/l; range, 300–1435 nkat/l) was significantly greater ($p < 0.05$) than that in those with undetectable free GE (median, 215 nkat/l; range, 39–1157 nkat/l). In UC patients the difference in total GE concentration between those with detectable free GE (median, 532 nkat/l; range, 55–1550 nkat/l) and those with undetectable free GE median, 162 nkat/l; range, 44–432 nkat/l) did not reach statistical significance.

DISCUSSION

When the procedure is standardized and supervised by an experienced nurse, whole-gut lavage with a polyethylene glycol-based isotonic solution is essentially a whole-gut perfusion system. Once the gut has been cleansed, the concentrations of various proteins in WGLF remain steady over serially timed samples, and the first clear sample is representative of the rest (unpublished data, and J. Sallam, Ph.D. thesis, University of Edinburgh, 1995). The clear fluid passed per rectum is suitable not only for detection of antibodies, as first described by Gaspari et al. (15), but also for cytology (3–6), for investigations of plasma protein leak (16) and bleeding (17) into the gut, and for studies of pro-inflammatory and immunoregulatory cytokines. Abundant material for study becomes available when gut lavage is used for bowel cleansing before clinically indicated diagnostic imaging or endoscopic tests, and since the technique is relatively non-invasive, healthy control volunteer subjects can also be studied. This approach is proving particularly useful for the investigation and analysis of gut inflammation.

Neutrophil infiltration into the tissues, migration into the gut lumen, and activation can be investigated in several ways—by histology and immunohistochemistry of mucosal biopsy specimens, by using radiolabelled leucocytes (18, 19), and by cytologic examination of whole-gut lavage fluid (6). The use of gut lavage has the advantage that both the relatively inaccessible small bowel and the colon can be studied quantitatively and objectively by cytology. However this test requires fresh specimens and is very labour-intensive. We have therefore developed and evaluated a biochemical assay specific for the neutrophil granule protein GE.

Some of the ELISAs for GE measure the enzyme as a complex with α_1-antitrypsin (20). The concentration of α_1-antitrypsin in WGLF is normally very low and is affected by disease (16), and so the ELISA for GE was unsuitable for our purposes. We therefore used a different technique, independent of the presence or concentration of α_1-antitrypsin. GE is released from the cells by sonication, then assayed by using a chromogenic substrate (L-pyroglutamyl-L-prolyl-L-valine-*p*-nitroanilide) specific for GE and a colorimetric method. The handling of the WGLF sample after collection differed from the procedure that we have previously described. For immunoassays, including IgG measurements, WGLF samples are filtered and treated with a series of protease inhibitors. Such specimens have, of course, been depleted of cells, including granulocytes, at the filtration stage, and GE cannot be detected by our method when protease inhibitors are present. Thus, total GE measurements were made on sonicated but otherwise untreated WGLF specimens. Free GE in the fluid can also be measured in filtered but otherwise untreated specimens, thus providing some information on cell activation.

We have previously shown that in patients with high cell counts in WGLF, the cells are predominantly neutrophils, and we suggested the term 'luminal neutrophilia' to describe this (6). The present study shows that WGLF GE concentration correlates significantly with cell count, confirming that GE is an excellent surrogate marker for luminal neutrophilia. Results of GE assays in this large series confirm and extend our cytologic findings that most, but not all, patients with luminal neutrophilia have IBD. Further analysis (6, and unpublished observations) is showing that when stratified separately by disease distribution (on the basis of X-ray and

endoscopic findings) and by disease activity (on the basis of concentration of IgG in WGLF), luminal neutrophilia is a feature of active ulcerative colitis and active colonic Crohn's disease but is not normally present in patients with active Crohn's disease of the small bowel.

Neutrophils and GE are also present in the WGLF from patients with diseases other than IBD, particularly in those whose clinical backgrounds were compatible with neutrophil migration through the gut wall. Acute radiation proctocolitis and diverticulitis are conditions obviously associated with pus present on the mucosa. A pericolic abscess communicating with the lumen may discharge pus, which is the most likely explanation in our patient with carcinoma of the colon with increased GE level. NSAID use has been shown to be associated with isotope-labelled leucocyte excretion into the lumen, probably from small intestine (21); two of our patients with markedly increased WGLF GE but normal IgG concentrations were taking NSAIDs.

There were a few IBD patients in whom results of total and free GE assays showed the presence of both cell-bound and cell-free GE in WGLF, indicating neutrophil degranulation; other IBD patients had cell-bound (or, strictly speaking, particulate) GE only. Total GE levels were significantly higher in the former group, but disease activity, as measured by WGLF IgG concentration, was similar in those with and without free GE.

Further studies with WGLF may help to elucidate the stimuli for granulocyte migration into the gut in different clinical situations. Various molecules that may act as chemoattractants, including interleukin (IL)-8, bacterial peptides such as formyl-methionyl-leucyl-phenylalanine (FMLP), leukotriene-B4, complement factors such as C5a, and platelet-activating factor (PAF), have been recently reviewed (22). The WGLF method can be used to investigate these possibilities. Preliminary studies of cytokines in WGLF suggest that IL-1 and IL-8 are present in lavage fluid from IBD patients (unpublished data). Evidence that IL-8 may be implicated in the pathologic lesions of IBD comes from rectosigmoid perfusion studies, which show that myeloperoxidase released from neutrophil granules correlates with IL-8 in the perfusate in ulcerative colitis (23). Furthermore, in active IBD, increased IL-8 content of affected colonic mucosa correlates with mucosal neutrophil counts (24).

In conclusion, the whole-gut lavage technique is a new, relatively non-invasive clinical method for detecting and investigating intestinal immunity and inflammation. Since the method may be readily combined with preparation for radiologic or colonoscopic investigation of the bowel, material from patients with gut lesions other than IBD may readily be studied to determine the specificity of particular immunologic variables for a defined pathologic condition. By using this new clinical test, it should be feasible to study the regulatory mechanisms underlying anatomic differences between small- and large-bowel diseases and the effects of pharmacologic agents such as steroids, antibiotics, and NSAIDs, and to objectively define therapeutic end points such as remission of gut inflammation.

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